Dietary, lifestyle and genetic determinants of homocysteine, and its relation with coronary heart disease

Angelika de Bree

Dietary, lifestyle and genetic determinants of homocysteine, and its relation with coronary heart disease

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General Introduction

Introduction

Cardiovascular diseases are a major public health problem in affluent countries. In 1999 about 36% (or ~50,000) of all deaths in the Netherlands were due to cardiovascular diseases. In comparison, about 27% was due to cancer, indicating that vascular diseases are the most important cause of death in the Netherlands (1).

Cardiovascular diseases can roughly be classified into three categories: 1) coronary heart disease (CHD), 2) cerebrovascular accidents (CVA), and 3) other vascular diseases. In CHD the coronary arteries that supply blood to the heart, are blocked and in CVA the arteries that supply blood to the brains are obstructed. The category "other vascular diseases" comprises occlusions of peripheral arteries or veins, and congenital, infectious and rheumatoid heart diseases. Of all cardiovascular diseases, CHD is the most prevalent one (1).

Blockage of the coronary arteries often begins with atherosclerosis. This is characterized by the deposition of cholesterol, cellular waste products, calcium and other substances in the inner layer of the arterial wall, together with the formation of connective fibrous tissue. This is called an atherosclerotic plaque. If plaques grow large enough they significantly reduce or obstruct the blood flow through an artery. They can also become fragile and rupture. Plaques that rupture induce the formation of blood clots (thrombosis). These clots may locally block the blood flow or break off and travel to other parts of the body where they may occlude other arteries or veins (2).

A high blood pressure, an unfavorable cholesterol profile (i.e. high total or LDL and low HDL cholesterol levels) and smoking explain the majority of all CHD cases. However, the search for other risk factors remains, as not all CHD cases can be explained by these established risk factors.

History of homocysteine as risk factor for vascular diseases

The process of identifying homocysteine as a possible risk factor for vascular disease already started in 1964. By that time Mudd and co-workers (3) showed that the accumulation of homocysteine in blood, and consequently in urine leading to homocystinuria, was due to deficiency of the enzyme cystathionine β-synthase (CBS). After this discovery, McCully (4) observed that a patient with CBS deficiency had comparable arterial damage as another patient with a different enzymatic abnormality that also led to homocystinuria. Since both abnormalities shared accumulation of homocysteine, McCully postulated that homocysteine, or one of its derivatives, was responsible for the arterial damage (4). This formed the basis for the hypothesis that moderate elevations of homocysteine in blood may be a risk factor for atherosclerosis in the general population (5). The first to test this hypothesis were Wilcken and

Wilcken in 1976 (6). They showed that patients with coronary artery disease suffered more often from an abnormal homocysteine metabolism than controls.

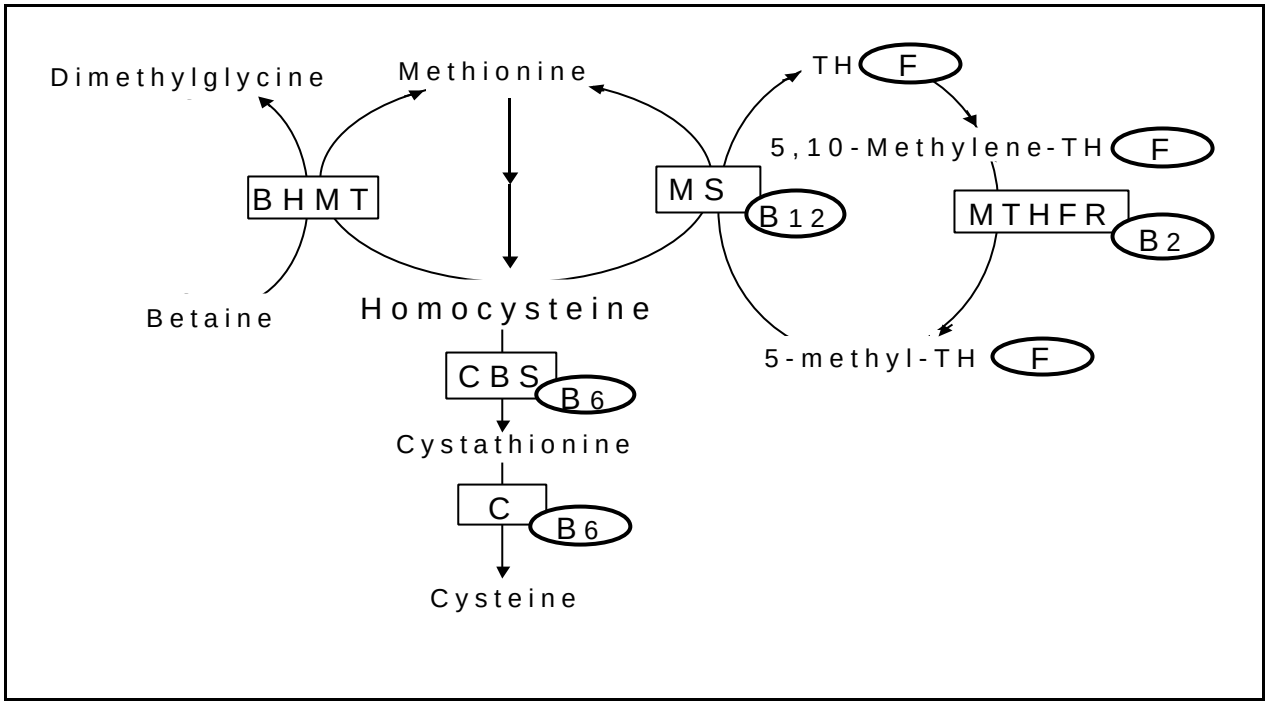
After the initial investigations, numerous experiments focused on the question how homocysteine might cause vascular damage. To date, no definitive mechanism has been identified. However, mostly *in vitro* experiments with very high homocysteine concentrations have identified the stimulation of atherosclerosis and thrombosis through adverse effects on coagulation pathways, platelets, endothelial cells, and vascular smooth muscle cells (7).

Homocysteine metabolism

Homocysteine is a sulphur-containing amino acid that is not used for the synthesis of proteins. Foods only contain traces of homocysteine. Homocysteine is formed when cells metabolize the essential amino acid methionine. The intracellular homocysteine concentration is precisely regulated and any excess is transported to plasma. In plasma approximately 99% is oxidized to disulphides. The vast majority of these disulphides is bound to proteins, only about 1% is reduced “free” homocysteine, unbound to proteins. The term total plasma homocysteine (conventionally abbreviated with tHcy) refers to all these forms of homocysteine in plasma (8). Roughly, moderate elevations in the tHcy concentration refer to fasting plasma concentrations >15-30 µmol/L, intermediate hyperhomocysteinemia refers to concentrations between 30-100 µmol/L, and severe hyperhomocysteinemia refers to concentrations >100 µmol/L (9).

Intracellular homocysteine can irreversibly be degraded to cysteine through the transsulphuration pathway that is mainly limited to cells of the liver and kidneys. The enzymes in this pathway, CBS and γ-cystathionase, are both dependent on pyridoxal-5’-phosphate, a biologically active form of vitamin B6, as co-factor. Homocysteine can also be remethylated to methionine, by the enzyme methionine synthase (MS). This enzyme uses methyl-cobalamin (a biologically active form of vitamin B12) as co-factor. The methyl-group for the latter reaction is donated by 5-methyl-tetrahydrofolate (5-methyl-THF). This form of folate is produced by the enzyme 5,10-methylene-tetrahydrofolate reductase (MTHFR). MTHFR in turn uses a biologically active form of vitamin B2 (flavin adenine dinucleotide) as co-factor (10,11). In an alternative remethylation route, which is also mainly restricted to the liver and kidney, betaine is used as the methyl-donor by the enzyme betaine-homocysteine methyltransferase (BHMT) to remethylate homocysteine (10). An overview of the homocysteine metabolism is presented in figure 1.

Figure 1 Simplified intracellular homocysteine metabolism



Explanation of abbreviations: BHMT=betaine-homocysteine methyltransferase, CBS=cystathionine β-synthase, C=γ-cystathionase, MS=methionine synthase, MTHFR=5,10-methylene-tetrahydrofolate reductase, TH=tetrahydro, F=folate, B2=vitamin B2, B6=vitamin B6, B12=vitamin B12.

Disturbances in intracellular homocysteine metabolism lead in most cases to elevated tHcy levels. Genetically determined functional deficiencies of enzymes in homocysteine metabolism, like deficiency of CBS, can have an extremely large impact on the tHcy concentration. On the population level these inborn errors of homocysteine metabolism are not important causes of elevations in tHcy concentrations, because they are rare; homozygous CBS deficiency is the most common inborn error and has a prevalence of about 1:335,000 (12,13). Possible tHcy-determinants on population scale are evaluated in the next section.

Determinants of homocysteine levels in the general population

Knowledge of factors that determine the tHcy concentration in the general population enables the identification of high-risk groups in which specific preventive measures can be taken. In addition, some determinants may be modifiable, which offers the possibility for public health messages describing how favorable changes in diet and/or lifestyle will lower tHcy levels. This could have a beneficial effect on the incidence of vascular diseases, assuming a causal relation.

One objective of this thesis is to identify tHcy-determinants in the general Dutch adult (20-65 years) population. We use the term “determinants” throughout this thesis to indicate factors that are associated with the tHcy concentration, not necessarily to indicate causal relations.

The following paragraphs give the state of the art with regard to the knowledge on tHcy-determinants in general populations at the beginning of this PhD-project.

Age and sex

Increasing age and male sex are associated with a higher tHcy concentration (14,15). The difference between the sexes could be due to the influence of sex hormones. It may also be due to larger muscle mass in men, since the formation of muscles is associated with the simultaneous formation of homocysteine in connection with creatine/creatinine synthesis (16). Part of the relation with age in women might be explained by the menopause, since the tHcy concentration was found to be higher in post-menopausal women compared with pre-menopausal women (14,17).

Dietary factors

In the early 90’s it was shown in intervention trials that supplemental folic acid, the synthetic form of folate in supplements, protected pregnant women from having a child with a neural tube defect (18,19). In the same period evidence accumulated that folic acid could lower tHcy levels of subjects with moderately elevated concentrations (20,21).

Besides folate, other B-vitamins are involved in homocysteine metabolism (see figure 1). At the time of the start of the project described in this thesis, inverse associations between the dietary intake of vitamin B6, B12 and folate and plasma tHcy levels were reported in a general population of elderly (22). However, no information was available on dietary intake of B-vitamins and their relation with the tHcy concentration in adults (20-65 years). Furthermore, no study investigated the relation between vitamin B2 intake and the tHcy concentration.

Genetic variants and gene-diet interactions

In addition to the rare inborn errors that lead to very high tHcy levels (12,13), there are genetic variants (polymorphisms) that have a less pronounced effect on the activity of the enzyme they encode for. The only polymorphism that has been linked to elevated tHcy concentrations is the 677 C>T mutation in the gene encoding for MTHFR (23). The homozygous mutant form (TT) is relatively prevalent in the Dutch general population (5-8% (24,25)). Higher tHcy levels were especially seen in TT subjects with a sub-optimal plasma folate status (26). This was the first identified gene-nutrient interaction influencing tHcy levels. Because of the role of the MTHFR enzyme in homocysteine and folate metabolism, it is also of interest to investigate whether the 677 C>T polymorphism has an effect on the relation between folate

intake and the plasma folate concentration, and on the relation between folate intake and tHcy level.

Lifestyle factors

One study based on a large population of adults from Norway investigated the association between the tHcy concentration and several lifestyle factors. Smoking (27,28) and coffee consumption (28,29) were associated with higher tHcy levels. Tea consumption was not associated with the tHcy concentration (29). Physical activity (27) and alcohol consumption (30) were inversely associated with the tHcy concentration. Confirmation of these results in other population-based studies is needed. Furthermore, the finding of an inverse relation between alcohol consumption and tHcy level is remarkable and needs further clarification, as there is also evidence that alcoholics have very high tHcy levels (31).

Biological CHD risk factors

Several studies concluded that an elevated tHcy level is an independent risk factor for CHD (32), though associations of the tHcy concentration with established biological risk factors have been demonstrated. The study from Norway (27) mentioned above reported a positive association of the tHcy concentration with total cholesterol level, diastolic and systolic blood pressure. A U-shaped association between the body mass index (BMI) and tHcy level was no longer present in multivariate models. Waist circumference might be a better indicator for abdominal fat than BMI and thus might be more relevant for CHD (33). The relation between HDL cholesterol level and the tHcy concentration was not investigated. To identify potential confounding factors in studies that quantify the risk of raised levels of tHcy for CHD, more knowledge on the associations between biological CHD risk factors and the tHcy concentration is needed.

Epidemiological studies on homocysteine and CHD

In 1995, Boushey *et al* (32) reviewed the available studies on tHcy concentrations and cardiovascular diseases. Most of these initial investigations had a retrospective or cross-sectional study design and consistently suggested a strong positive relation between moderate elevations in tHcy concentration and risk of cardiovascular diseases (32). A major limitation of these study designs is that it can not be excluded that the increased tHcy levels are the result rather than the cause of the disease, because blood samples for these studies are typically taken after the diagnosis of the cardiovascular event. Prospective studies provide stronger evidence for a causal role of elevated tHcy levels in the etiology of cardiovascular diseases, as collection of blood took place before the event.

When the present PhD-project started in 1997, there were only a few studies with a prospective design relating elevated tHcy levels to cardiovascular diseases (34). Even fewer studies considered CHD as an endpoint (35-39). The Physicians' Health Study found that after 5 years of follow-up, men with a high tHcy concentration (defined as >95th percentile of the tHcy distribution) had a three-fold higher risk to die of a myocardial infarction relative to men with lower tHcy levels (defined as <90th percentile) (35). However, after extending the follow-up period from 5 to 7.5 years, high tHcy levels were no longer associated with an increased risk of myocardial infarction (36). In another study (37) patients with vascular diseases were followed to investigate whether higher tHcy levels were related to recurrence of vascular diseases, including CHD. This was found to be true, as patients with elevated tHcy concentrations more often demonstrated clinical progression of CHD, when compared with patients with a tHcy level considered normal (37). Of the 2 available population-based investigations (38,39), a Finnish study of Alfthan *et al* (39) found that each 5 µmol/L increase in tHcy concentration was not associated with an increased risk of myocardial infarction (relative risk (RR)=1.03, 95% confidence interval (CI)=0.66-1.53 (40)). Conversely, a Norwegian study of Arnessen *et al* (38) found that each 5 µmol/L increase in tHcy concentration was progressively associated with the risk of myocardial infarction (RR=1.41, 95% CI=1.06-1.88 (40)). Both studies included adult men and women free of baseline myocardial infarction. An important difference may be that the follow-up period of the Finnish study was 9 years, whereas the follow-up period in the Norwegian study was only 4 years.

Since 1997 the results of more prospective studies have become available (40-43). The RR estimates from these studies are smaller, and often not statistically significant, than the RRs found in earlier cross-sectional and retrospective studies (44). This has increased the doubt on whether an increase in tHcy concentration is causally related to the development of CHD (44,45). Nevertheless, a meta-analysis taking the results of most prospective studies together, shows an overall positive association between the tHcy concentration and the risk of CHD (40). Yet, the inter-study differences are sometimes large, which may derive from different lengths of follow-up and from differences in the composition of the study populations. In this thesis we will further explore the prospective relation between the tHcy concentration and the risk of CHD mortality in men and women aged 20-59 years.

Outline of the thesis

The main objectives of this thesis are to provide information on the determinants of plasma total homocysteine (tHcy) concentrations, and to prospectively quantify the risk of elevated tHcy levels for CHD mortality. Given the tHcy-lowering effect of folate in supplemental form, we considered folate an important potential tHcy-determinant. Therefore we start this thesis with a

review on folate intake (**CHAPTER 2**). In this review we describe the 1997 knowledge with regard to actual, recommended and desired folate intake in relation to obtaining and maintaining optimally low tHcy levels.

In **CHAPTER 3** we describe the tHcy distribution of the general Dutch adult population. This information is of value for public health authorities that want to estimate the possible public health burden of the tHcy concentration in the population. To accurately estimate this burden, the tHcy measurement needs to be valid and precise, thus issues that affect accuracy are described. Finally, the effects of age and sex on the tHcy distribution are shown.

As will become clear from chapter 2, the knowledge on the relation between B-vitamin intake and the tHcy concentration is mainly restricted to results from intervention trials using supplements. In **CHAPTER 4** we will investigate the relation between *dietary* intake of folate, and also of the other B-vitamins involved in homocysteine metabolism, and the tHcy concentration in detail.

Like described earlier, the MTHFR 677 C>T genotype modifies the relation between plasma folate and tHcy concentrations. In **CHAPTER 5** we complement this available information with the effect of the genotype on the relations between 1) folate intake and plasma folate concentration and 2) folate intake and tHcy concentration.

To clarify and to confirm or reject some of the relations between lifestyle and tHcy level we describe the relation between reported smoking, physical activity, coffee, tea and alcohol consumption and the tHcy concentration in **CHAPTER 6**. To explore whether one particular type of alcohol beverage was especially associated with the tHcy concentration, we analyzed its relation with beer, wine and spirits in **CHAPTER 7**.

To indicate potential confounders for the relation between tHcy level and the risk of CHD, we describe how total and HDL cholesterol level, diastolic and systolic blood pressure, and waist circumference, are associated with the tHcy concentration in **CHAPTER 8**.

We prospectively examined whether an elevated tHcy concentration is a risk factor for CHD mortality in the general Dutch population aged 20-59 years in **CHAPTER 9**. This thesis concludes with a general discussion of our findings in **CHAPTER 10**.

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2

Folate intake and the relation with homocysteine: the 1997 state of the art

Objectives: To describe and compare recommended, actual and desired folate intake in light of obtaining and maintaining optimally low plasma total homocysteine (tHcy) concentrations in European adult populations.

Design: Review of dietary recommendations for folate, food consumption surveys providing information on folate intake, and intervention and observational studies relating folate intake to tHcy concentrations.

Results: The recommended intake in different countries varies between 200-300 µg/d (men) and 170-300 µg/d (women). In Europe, mean dietary folate intake in adults is 291 µg/d (range 197-326) for men and 247 µg/d (range 168-320) for women. A daily dose of 650 µg supplemental folic acid is desired to normalize elevated tHcy levels and a dietary folate intake of at least 350 µg/d is desired to obtain and maintain optimally low tHcy levels in the adult population in general.

Conclusions: Mean dietary folate intake in Europe is in line with the recommendations, but is not in agreement with the desired dietary intake of >350 µg/d. This level of intake is only reached by a small part of the studied European populations. More research is required 1) to establish the lowest effective dose of supplemental folic acid to normalize high tHcy levels, 2) to determine the lowest effective dose of dietary folate to optimize tHcy levels in the normal range, and 3) on the bioavailability of folate either naturally or artificially present in foods, which will enable the choice of a strategy to achieve desired folate intakes in the general population.

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Introduction

Folate is a B-vitamin, which acts as a coenzyme in several single carbon transfer reactions to synthesize components of DNA, RNA, and amino acids. The term folate describes the group of derivatives containing the parent molecule pteroylmonoglutamic acid, which exhibits biological activity. In food, folates are mainly present as reduced tetrahydrofolate polyglutamates. Folic acid is the synthetic fully oxidized form of pteroylmonoglutamic acid, which is present in supplements and does not occur naturally in significant quantities.

Folic acid is classically used to treat deficiency symptoms like megaloblastic anemia arising during pregnancy or infancy. Folate deficiency can also originate from a poor dietary intake or from absorption disorders like celiac disease. Folic acid should never be used to correct the macrocytic anemia due to vitamin B12 deficiency (1). During the past 30 years new functions of folate have been discovered. In 1965 Hibbard and Smithells postulated that folate deficiency might be associated with imperfect closure of the neural tube (2). At present there is solid evidence that folic acid supplementation during the periconceptional period significantly reduces the risk of giving birth to a child with a neural tube defect (3). More recent findings indicate that supplementation with folic acid decreases plasma total homocysteine levels (tHcy) (4-7). Furthermore, there are indications that a high dietary folate intake is associated with lower tHcy levels (8,9). These are significant findings, since elevations in tHcy levels have been associated with a graded increased risk of vascular diseases, independently of established cardiovascular risk factors (10). Finally, there is a growing number of studies showing that a high folate intake is related to a reduced risk of certain types of cancer, like colorectal cancer (11) but thus far no conclusive evidence on this relation is available (12).

From a public health perspective these new insights on the role of folate are important. Consequently, this has raised concern about the folate intake of the general population both by the scientific community (10,13,14) and health authorities (15-20). Scientists have proposed to increase the current recommended intake levels for folate (8,9,21,22) and this has resulted in a discussion on possible strategies to raise the folate intake of the general population (10,14,23,24).

This review describes and compares the recommended, actual and desired folate intake with regard to obtaining and maintaining optimally low tHcy levels. We concentrate on the general adult population (age 18-64 years).

Folate recommendations

Recommended dietary intake (RDI) is a general term to describe the intake level estimated to be sufficient to cover the needs of the majority (97.5%) of the healthy population. The recommendation should provide a margin of safety to allow for periods of decreased intake, increased utilization, individual variability and bioavailability of folates in foods. The RDI can be used to evaluate intake figures derived from nutritional surveys e.g., to detect groups with a low intake (25).

Within the European Community, France, Germany, Italy, Ireland, the Netherlands, Portugal, Spain and the UK have composed their own set of RDIs. Belgium uses the recommendations of several countries (the USA, the UK, the Netherlands and France) and Greece uses those of the FAO/WHO and the USA. The Northern European countries Denmark, Sweden, Norway, Finland and Iceland have established the Nordic Nutrition Recommendations. Beyond the European community, Bulgaria, the Czech and Slovak republic, Hungary, Poland, and Russia all have their own recommendations (26).

The most up to date recommendations for dietary folate intake in Europe are those of France, Germany, the Netherlands, and the UK. These recommendations and those of the FAO/WHO and the USA are summarized in table 1. The recommendations for males vary from 200 to 300 µg/d and for females from 170 to 300 µg/d. Despite this variation between countries, the most frequently recommended level of dietary folate intake is 200 µg/d for men and women.

The variation in recommended intake levels is due to the fact that each country has its own approach, correction factors, and publications on which the recommendations are based. In general, two manners to establish the RDI for folate can be distinguished. The first approach is to determine a minimum requirement that is necessary to maintain normal metabolic processes or to cure clinical and biochemical symptoms of folate deficiency. This minimum amount is then increased in order to correct for bioavailability and individual variation. The second method is to evaluate the average dietary folate intake of a population with a known biochemical folate status. With the information on folate intake and the prevalence of e.g. low red blood cell folate status, an RDI value can be established which assures a sufficient folate intake for the whole population. The committees which constituted the RDI for the FAO/WHO, the USA, the UK, and France combined both approaches. The RDIs of the Netherlands and Germany were solely based on the first approach. The estimated minimum requirements and the correction factors are also given in table 1.

Table 1 Minimum requirements, correction factors and recommended dietary folate intakes (in µg/d) for adults (≥ 18 years)

Organization/ Country	Minimum requirements	Correction for:		Recommendations	
		individual variation	bioavailability	Men	Women
FAO/WHO (27)	60	+15%	+70%	200	170
USA (28)	60	+30%	+50%	200	180
UK (29)	100	+?	+50%	200	200
The Netherlands (30)	50	+200-300%	+50%	200-300	200-300
France (31)	50-100	?	?	300	300
Germany (32)	150	nc	+50%	300[a]	300[a]

Explanation of abbreviations and symbols: +?=corrections were made, but it is unknown to which extent; ?=unclear whether corrections were made; nc=no corrections were made; a=the actual German recommendation, for both men and women, is 460 µg/d. This amount is based on the folate content of "raw" material. In this table the actual recommended folate intakes are summarized, these have been corrected for the estimated 35% loss of folate during preparation and cooking (32).

Folate intake

Folate is present in a wide range of foods. Particularly rich in folate are liver, yeast, green leafy vegetables, legumes, and fruits like mandarins and grapefruits (33). These foods are not necessarily the predominant contributors to the folate intake. Major contributors to the folate intake in the Netherlands, the UK, and Ireland are vegetables, bread and potatoes, responsible for respectively 15-22%, 12-22%, and 10-15% of the total daily intake (34-36).

Folate intakes of adult populations in Europe are shown in table 2. For this table large recent national and regional nutritional surveys have been used. When more than one survey per country was available, the most representative study is shown. The age categories are tabulated in the format in which they were shown by the original survey. This means that for some countries the folate intake of subjects under 18 years are presented as well.

The highest folate intake was reported in the Parisian area in France i.e., median folate intake of men was higher than 400 µg/d, and that of women was higher than 350 µg/d (37). The lowest folate intakes were found in British, Swedish, and Irish females, and in Irish men aged 40 years and older. In these populations the median or mean folate intakes were lower than 200 µg/d (35,36,38). Overall, the weighed mean folate intake was 291 µg/d (range: 197-326 µg/d) for men and 247 µg/d (range: 168-320 µg/d) for women.

Differences in folate intake may partly reflect differences in dietary habits among the European countries. For example, the higher folate intake in France, but also in Spain and Portugal, might originate from the Mediterranean diet consumed in these populations. This diet

consists of a higher portion of folate-rich foods such as vegetables, fruits, and whole grains compared with Northern European diets (39).

Methodological differences between studies are another source of variation in folate intake. The different methods used to collect dietary information all have their own advantages and disadvantages that can potentially have led to an over- or underestimation of the habitual intake (24). Another methodological issue is the unreliability, or even the lack, of folate content data in food composition tables. This problem is thought to lead to an underestimation of the actual folate intake (40). Most surveys did not use food composition tables based on internationally standardized methods to determine the folate concentration in food, instead tables based on various techniques to determine the folate content of foods commonly eaten within that country were used. An exception is the Dutch survey, which used the recent edition of the McCance and Widdowson's (33). This food composition table gives a complete overview of the folate content of a broad range of foods and all food samples have been analyzed with the same method. The analysis of the folate content of food is difficult. The principle forms of folate in food are reduced pteroylpolyglutamates. High-performance liquid chromatography (HPLC), ligand binding methods or microbiological assays with the *Lactobacillus casei*, can measure these (41). The accuracy of the analysis is highly dependent on the preparative steps, consisting of the enzymatic deconjugation and the extraction of the polyglutamates. Some previously used extraction techniques might not have yielded a complete extraction of folates from the food sample matrix (42). All these methodological differences may partly account for the different folate intake estimates in the presented studies.

Factors that do not seem to explain differences in average folate intake level among the surveys are vitamin supplement use and fortification with folic acid. For instance, although foods fortified with folic acid and vitamin preparations were included in the intake values of the Irish survey, folate intake was still low (36). In contrast, the intake of folate was much higher in France excluding vitamin supplements and fortified products (except for some special formulas like dietetic products) (37).

Table 2 Daily intake of folate (µg/d) in adult populations in Europe

				Men	
				Intake	
Country, reference	Method	Age	N	Mean	Median
France	dietary history	18-29 y	84	-	389
Hercberg *et al*, 1993		30-39 y	94	-	408
(37)		40-49 y	72	-	399
		50-64 y	76	-	453
		total, 18-64 y	326		R:236-674
Spain	24 hr recall & food	18-34 y	360	312	290
Serra Majem *et al*, 1996	frequency	35-49 y	242	326	289
(43)	questionnaire	50-64 y	218	315	288
		total, 18-64 y	820	317	R:130-615
Portugal	24 hr recall	15-19 y	631	306	-
Gonçalves Ferreira *et al*, 1988		20-49 y	2188	295	-
(44)		50-64 y	1016	307	-
		total, 15-64 y	3835	300	
The UK	7 d weighed	16-24 y	214	302	285
Gregory *et al*, 1990	record	25-34 y	254	317	303
(35)		35-49 y	346	321	308
		50-64 y	273	300	289
		total, 16-64 y	1087	311	R:138-600
Denmark	7 d dietary record	15-18 y	68	295	295
Andersen *et al*, 1996		19-24 y	85	313	281
(45)		25-34 y	142	282	290
		35-44 y	135	329	286
		45-54 y	147	291	288
		55-64 y	140	313	287
		total, 15-64 y	717	304	R:142-603
The Netherlands	2 d dietary record	16-18 y	128	274	255
Bausch-Goldbohm *et al*, 1995 (34)		19-21 y	111	289	281
		22-49 y	1306	293	281
		50-64 y	405	299	282
		total, 16-64 y	1950	293	R:131-510
Germany	7 d dietary record	15-18 y	592	320	285
Adolf *et al*, 1994, 1995 (46)		19-24 y	1182	301	269
		25-50 y	4974	282	256
		51-65 y	1699	284	261
		total, 15-65 y	8447	288	R:123-714

Explanation of abbreviation: R=range, derived from lower (2.5 or 5th) and upper (95 or 97.5th) percentile figures.

Women				
	Intake		Conducted in	Comments
N	Mean	Median		
127	-	350	1998	Representative sample for Parisian area in France.
102	-	379		
90	-	375		Intake from dietary sources only.
97	-	391		
416		R:196-652		Fortifying general food products with folate is not allowed in France, except for infant formulas and specialized dietetic products.
431	282	257	1992-1993	Representative sample for Catalan area in Spain.
323	317	291		
260	320	301		Not clear if supplement use is included.
1014	303	R:116-584		In 1997, fortification with folic acid was not allowed in Spain.
640	257	-	1980	Representative sample for Portugal.
2571	263	-		
1237	273	-		Not clear if supplement use is included.
4448	265			Fortification of breakfast cereals was allowed in 1997, but fortified products were not included in this study.
189	198	194	1986-1987	Representative sample for the UK.
253	206	198		
385	220	212		Intake from dietary sources only.
283	218	214		
1110	213	R:84-383		Fortification of foods was allowed in 1997, but fortified products were not included in this study as fortification was just being introduced: 1986-1987.
84	265	234	1995	Representative sample for Denmark.
100	244	220		
161	266	232		Intake from dietary sources only.
158	234	239		
155	248	248		In 1997, fortification with folic acid was not allowed in Denmark.
128	241	240		
786	249	R:138-469		
125	227	215	1992	Representative sample for the Netherlands.
107	243	236		
1493	234	224		Intake from dietary sources only.
545	249	242		
2270	238	R:108-390		In 1997, fortification with folic acid was not allowed in the Netherlands.
730	260	233	1985-1988	Representative sample for Germany
1504	251	227		
5304	242	219		Not clear if supplement use is included.
2103	249	230		
9641	246	R:98-600		In 1997, fortification with folic acid was not allowed in Germany.

Table 2 continued

Country, reference	Method	Age	N	Men Intake Mean	Men Intake Median
Sweden	7 d dietary	15-18 y	56	265	-
Becker, 1992 (38)	record	19-29 y	152	250	-
		30-44 y	243	225	-
		45-64 y	251	215	-
		total, 15-64 y	702	230	
Ireland	dietary history	18-24 y	51	255	237
Lee & Cunningham, 1990 (36)		25-39 y	85	244	238
		40-59 y	87	197	190
		total, 18-59 y	223	228	

Explanation of abbreviation: R=range, derived from lower (2.5 or 5th) and upper (95 or 97.5th) percentile figures.

Desired folate intake in light of obtaining and maintaining normal homocysteine levels

B-vitamins play an important role in homocysteine metabolism. Folate is the methyl-donor for the remethylation of homocysteine to methionine. Furthermore, vitamin B2, B6 and B12 are cofactors of enzymes in homocysteine metabolism (47). In case of a B-vitamin shortage, intracellular homocysteine will accumulate and the excess is transported to plasma. Elevations in the plasma tHcy concentration are associated with an increased risk of vascular diseases (10). The mechanism underlying this association is not completely understood, but homocysteine presumably stimulates atherosclerotic as well as thrombotic processes (48).

Because of the substrate donor function of folate in homocysteine metabolism, it may have the potential to prevent vascular diseases. This hypothesis is supported by the observations that low serum folate levels were associated with an increased risk of ischemic stroke (49) and coronary heart disease (50). However, randomized trials are needed to provide definitive proof for this association (51).

There is no consensus considering the definition of normal or elevated tHcy levels. Reported normal plasma values in healthy blood donors ranged from 7.9 to 9.3 µmol/L (48). Values >16.3 µmol/L are commonly considered indicative of mild hyperhomocysteinemia (5-7) although others have used levels >13.6 µmol/L (52), >14 µmol/L (8), and >15 µmol/L (53).

	Women			
	Intake		Conducted in	Comments
N	Mean	Median		
69	192	-	1989	Representative sample for Sweden.
193	205	-		
251	185	-		Not clear if supplement use is included.
253	195	-		
				In 1997, fortification with folic acid was not allowed in Sweden.
766	194			
54	169	157	1990	Representative sample for Ireland.
122	182	175		
111	168	160		Supplement use is included.
287	174	-		Fortified products like breakfast cereals were included in this study, as fortification was allowed since 1987.

In the following paragraphs we evaluate studies that provide evidence on the amount of folate necessary to obtain and maintain normal tHcy levels. One of the first intervention trials, in which the effect of folic acid supplementation on tHcy levels was investigated, was performed in 1988 (4). The authors showed that daily supplementation with 5000 µg folic acid for two weeks, reduced the tHcy concentration with 52% in a group of men and women (n=42) with slightly elevated tHcy concentrations. This reduction was not observed in the groups supplemented with vitamin B6 (40 mg/d) or B12 (1 mg/d). Unfortunately, this study did not include a control group (4).

A daily combined vitamin supplement containing folic acid (1000 µg), vitamin B6 (12.2 mg), and vitamin B12 (0.4 mg) for six weeks in mild hyperhomocysteinemic men (n=26), normalized tHcy levels (<16.3 µmol/L). Doubling the vitamin doses for two weeks, after the initial six weeks, did not result in a further decrease in tHcy levels (5). The same supplement was used in another trial (6) with 20 hyperhomocysteinemic men. After 6 weeks of supplementation, an 18-week washout period followed. Based on the tHcy concentration in week 24, the group was divided in men with normal (<16.3 µmol/L) and elevated tHcy levels (>16.3 µmol/L). In the subsequent experimental period, the group with the normal levels received advice to include more folate-rich foods in their diet. Adherence to these guidelines should have provided 200 µg folate/d. At the end of the experiment (week 48) these men still had normal tHcy levels (6).

Ubbink *et al* (7) also conducted a placebo-controlled trial in which the individual effects of vitamin B6 (12.2 mg), B12 (0.4 mg) and folic acid (650 µg) on mild hyperhomocysteinemia were compared with the effects of a combined supplement and a placebo. The study comprised 91 men with mild hyperhomocysteinemia (>16.3 µmol/L). No significant reduction in mean tHcy level was found in the placebo group or in the vitamin B6 group. In contrast, the groups supplemented with vitamin B12 or with folic acid, or with the three vitamins combined showed

reductions of 15, 42 and 50%, respectively. The reduction in mean tHcy level with the combined supplement did not significantly differ from the reduction established with folic acid alone. From these results the researchers concluded that the tHcy-lowering effect of the combined supplement was mainly due to folic acid.

Jacob *et al* (21) performed a controlled depletion-repletion-depletion-repletion study for 108 days in 12 non-smoking apparently healthy men. During the depletion periods the subjects were fed a diet providing ~25 µg/d of dietary folate. The tHcy levels increased significantly during depletion and did not normalize in the repletion phases, when the participants were given 74 µg supplemental folic acid/d and 25 µg dietary folate/d. The authors assumed that 74 µg folic acid equaled ~148 µg dietary folate (bioavailability of food folates is ~50% relative to folic acid). Thus, the total folate intake of ~173 µg/d was only slightly less than the USA RDI of 200 µg/d (table 1). Based on the lack of normalization of tHcy levels during the repletion periods, it was concluded that the RDI is not sufficient to obtain a normal tHcy concentration (21).

This conclusion is complemented with results of a controlled dietary trial in which was shown that an RDI of 200 µg/d is not sufficient to maintain normal tHcy levels (22). For a period of 70 days, a daily diet providing 30 µg dietary folate plus 170 µg supplemental folic acid (n=5), significantly increased tHcy levels as compared with baseline levels in apparently healthy women. The tHcy levels remained normal in the groups with a daily intake of 30 µg dietary folate plus 270 µg (n=6) and 370 µg (n=6) of supplemental folic acid.

In observational studies with population-based elderly (8) and population-based controls (9), the curve describing the relation between folate intake and the tHcy concentration, reached its nadir at a *total* dietary folate intakes of 350-400 µg/d (8,9). *Total* refers to folate from the diet and from supplements. Nevertheless, when only food folates were plotted against tHcy levels, the nadir was reached at the same amount of folate (P. Verhoef, unpublished data).

Taking all the evidence together, the lowest dose of folic acid that normalized elevated tHcy levels, was 650 µg/d (7). Once tHcy levels are normalized a daily dietary folate intake of 200 µg maybe sufficient to maintain this level (6). However, this contrasts the results of the trial in which 200 µg folate/d (of which 170 µg folic acid and 30 µg dietary folate) was not enough to prevent an increase in tHcy concentration (22). As these results were obtained with small numbers of subjects, in an uncontrolled setting (6) and without control groups (6,22), it might be safer to rely on the results of the observational studies. These indicate that a dietary folate intake of at least 350 µg/d is probably enough to maintain optimal low tHcy levels (8,9).

Discussion

Most health authorities recommend a daily intake of 200 µg dietary folate for both men and women (table 1). Nutrition surveys providing information on mean folate intake and the standard deviations, offer the possibility to estimate the proportion of the population with an intake below the generally recommended level of 200 µg/d. However, this is only allowed when folate intake is normally distributed, which is not the case evaluating medians and percentile figures. Nevertheless, it is still possible to make a rough estimate. For example, when median folate intake of a population equals the RDI, 50% of the population has an intake below the RDI. Using this strategy, table 2 indicates that the majority of the adults in the studied European countries meet the average RDI of 200 µg/d; weighed mean intake is 291 µg/d for men and 247 µg/d for women and median values lower than 200 µg/d are not frequently seen.

There appears to be a North-South gradient in dietary folate intake as the highest folate intake is observed in the Mediterranean area, and the lowest intakes are found in the UK, Sweden, and Ireland. In the latter countries a substantial part of the population does not meet the average recommended folate intake of 200 µg/d, which could indicate that the folate requirements for some individuals are not completely covered. However, it remains possible that the folate intake is underestimated due to unreliable food composition tables (40-42).

Comparison of actual intakes with the recommended intakes cannot be used to diagnose under-, mal- or over-nutrition of groups or individuals. This can only be done after the assessment of the clinical or biochemical status (26). The folate status can be determined by measuring the concentration of folate either in plasma or serum (reflecting recent folate intake), or in red blood cells (reflecting intake of the past 2-3 months) (54). Herbeth *et al* (55) determined the plasma and red cell folate concentration of a population of 710 adult men and women. They observed that of the subjects with a folate intake below the RDI (<200 µg/d), only 25% also had a low plasma folate concentration (<5 µg/L) or a low red blood cell folate concentration (<200 µg/L). One target of the RDI is to provide a margin safety to allow for periods of decreased intake, increased utilization, individual variability and bioavailability of folates in food (25). Therefore, these results (55) are in line with this margin.

RDIs are not established to indicate a level of desired intake. In this review we investigated a desired folate intake with respect to obtaining and maintaining an optimally low tHcy concentration. Optimally low tHcy levels are desired in light of the continuous positive graded association of the tHcy concentration with the risk of vascular diseases. However, the causality of this association is not yet proven, thus, we can not substantiate that the desired intake that we established would lead to a lower incidence of vascular diseases. Nevertheless, we have distinguished two levels: 1) 650 µg/d of *supplemental* folic acid for adults with elevated plasma tHcy levels; and 2) a *dietary* folate intake of at least 350 µg/d for the general adult population.

Achieving a dietary folate that is equally effective as 650 µg of folic acid would mean a dietary folate intake of 1300 µg/d, assuming that food folates are only for 50% bioavailable (relative to folic acid (56,57)). As the average actual folate intake is ~400% lower (table 2), it seems more realistic to achieve this desirable intake with supplements. There are, however, preliminary indications that lower doses of folic acid may also effectively lower tHcy levels (58,59).

To maintain normal tHcy levels, food folates alone may be effective (8,9). Table 2 indicates that only in the French survey, the majority of the population reached the desired dietary folate intake of at least 350 µg/d (median intake men ~410 µg/d, women ~370 µg/d) (37). We have to bear in mind, however, that the evidence for maintaining normal tHcy levels with dietary folate was derived from only two observational studies (8,9). Future results from randomized intervention trials might be able to provide evidence considering the lowest dose of dietary folate to obtain and maintain optimally low tHcy levels.

The discrepancy between the average actual folate intake and the desired folate intake asks for strategies to increase the folate intake. Is it realistic to stick to dietary advises, while the bioavailability of supplements is much higher? The difference in bioavailability of supplements and food is largely due to the fact that folic acid (pteroylmonoglutamic acid) can be absorbed directly, whereas food folates (mainly polyglutamyls) need to be enzymatically hydrolyzed to the monoglutamyl form before absorption (60).

The easiest way to increase folate intake may be consuming foods fortified with folic acid. Compared with folic acid supplements, fortified products are somewhat less bioavailable, depending on the characteristics of the carrier food (e.g., food matrix, food compounds that inhibit absorption, etc.). For example, relative to folic acid the bioavailability of fortified bread was 30%, of fortified maize 50%, and of fortified rice 60% (61). Nevertheless, the bioavailability of fortified products is higher than that of folates naturally present in foods, as indicated by preliminary results of an intervention trial performed by Cuskelly *et al* (62). They used five intervention groups all targeted to provide an extra 400 µg of folate/d, and one control group. After 3 months intervention, red blood cell folate increased only in the groups receiving supplements and fortified foods, compared with the control group. The group supplied with folate rich foods and the group receiving dietary advice did not show an increase in red cell folate. Weaknesses of this study were that the design did not allow control of the dietary intake and of food preparation methods, during which folate losses can easily occur (56). In addition, the intervention groups were very small: between 6 and 10 subjects per group (62). In conclusion, fortified foods are effective in optimizing the folate status, nevertheless, the shortcomings of the study design hamper the conclusion that natural food cannot effectively optimize the folate status. Consequently, more data on bioavailability of folate in foods is needed.

Conclusions

Current dietary folate intake of adults (291 µg/d for men and 247 µg/d for women) is in line with the average recommended level i.e., 200 µg/d. Elevated tHcy levels can be normalized with 650 µg supplemental folic acid/d. To maintain optimally low tHcy levels a dietary folate intake of at least 350 µg/d may be sufficient.

None of the most recent folate intake recommendations (table 1), proposes a desired dietary folate intake level of 350 µg/d. In light of optimally low tHcy levels, which may be associated with a lower incidence of cardiovascular diseases, a reconsideration of the current recommendations might be appropriate. Furthermore, the dietary surveys (table 2) show that only in the French population more than 50% of the population reaches a dietary folate intake >350 µg/d.

More research should focus on the lowest effective dose of supplemental folic acid to normalize initial high tHcy levels and on the lowest effective dose of dietary folate to maintain an optimally low tHcy level. In addition, more data is needed on the bioavailability of folate either naturally or artificially present in foods. This research will enable the choice of the optimal strategy to achieve desired folate intakes in the general population. In the meantime, it will be worthwhile to stimulate the consumption of foods naturally rich in folate, especially in Northern European countries where the folate intake is lower than 200 µg/d for more than 50% of the women of childbearing age and in men ≥40 years. Increasing the consumption of foods of plant origin is safe and will increase the intake of other beneficial nutrients and non-nutrients simultaneously.

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3

The plasma homocysteine distribution in the general Dutch adult population

The non-fasting plasma total homocysteine (tHcy) concentration was measured in a random sample of 3025 Dutch adults aged 20-65 years (main study). The positively skewed distribution had a geometric mean of 13.9 µmol/L in men and 12.6 µmol/L in women. Blood of the main study was not cooled or centrifuged immediately after drawing. A stability study (n=26) indicated that this could have resulted in a small (0.4 µmol/L) overestimation of the means. A comparative study (n=88), and a reproduction of these results in an entirely different population (n=213), showed a systematic difference in tHcy concentration of –2.4 µmol/L between our laboratory (Nijmegen, the Netherlands) and that in Bergen, Norway. With the information of the additional studies we provided precise and valid data of the Dutch tHcy distribution, from which we conclude the status in the Netherlands is worse than in other European countries. Furthermore, we showed that comparison of tHcy data is complicated unless the interlaboratory differences are known.

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Introduction

Accumulating evidence indicates that the plasma total homocysteine (tHcy) concentration is an independent risk factor for cardiovascular diseases (CVD) (1-8). Therefore, it is important that health authorities are provided with information on the possible burden that it might constitute in the general population. For this purpose knowledge on the tHcy distribution is necessary. This seems straightforward: provide a laboratory with plasma from a representative population and describe these data. However, some frequently disregarded problems can lead to an incorrect interpretation of the tHcy status.

The first problem emerges when a study is not designed to measure the tHcy concentration. Optimal blood sampling conditions, i.e. cooling or centrifuging whole blood directly after drawing (9), are necessary to obtain a valid tHcy measurement. Leaving whole blood at room temperature might artificially increase the tHcy concentration, which is already detectable after 1 h (10,11).

A second commonly overlooked problem is that the tHcy status cannot simply be evaluated by using reference values established in laboratories other than the laboratory where it was measured. This difficulty is due to the lack of a gold standard and of a (inter-)national standardization program for the measurement, which can result in large differences (9-15%) in the tHcy concentration of one sample measured in different laboratories (12,13).

The goal of this paper is to provide information on the tHcy distribution in the general Dutch adult population (main study), which has been unavailable up until now. To critically judge this distribution we performed a stability study and a comparative study, also described in this article.

Subjects and methods

Subjects

For the main study we used data from the population-based MORGEN study. This is a cross-sectional investigation into the prevalence of risk factors for chronic diseases as well as the prevalence of some specific chronic conditions, using self-administered questionnaires and a physical examination in a randomly selected sample of the Dutch population aged 20-65 years in three Dutch cities (Amsterdam, Doetinchem, and Maastricht) (14).

From the 19,066 subjects that participated in the MORGEN study during 1993-1996, 14,356 met our inclusion criteria (i.e. completed questionnaires, blood available for all aimed biochemical analyses, recorded time of drawing and centrifugation of blood). Of these, 7992 subjects were excluded because their whole blood had been stored at room temperature for

more than 1 h. This left us with a sample of 6364 subjects, out of which we drew an age and sex stratified sample of 3025 subjects. This sample did not differ from the total 1993-1996 MORGEN population with respect to established cardiovascular risk factors like total and HDL cholesterol level, blood pressure, social economic status, body mass index and smoking.

The stability study was performed in 1998 with 26 apparently healthy volunteers (14 men, 12 women, aged 24-57 years), working at the National Institute of Public Health and the Environment.

The comparative study was done in 1999 after the tHcy concentrations (measured in the Laboratory of Pediatrics and Neurology of the University Hospital in Nijmegen, the Netherlands) of the main study were available. We made sex-specific deciles and randomly drew 30 subjects (15 men and 15 women) out of the highest decile, the middle two deciles, and the lowest decile. From 2 subjects there was not enough plasma, for the remaining 88 subjects the tHcy concentration was also analyzed in the Laboratory of the Department of Pharmacology at the University of Bergen, Norway. To reproduce the results of our comparative study we used data (n=213) from another previously described study population (15). The fasting tHcy concentration of this population was measured in Bergen between 1992 and 1994, and re-analyzed in Nijmegen in 1999.

The external Medical-Ethical Committee of the TNO Toxicology and Nutrition Institute, which follow the guidelines of the Helsinki Declaration, has approved the studies described in this paper. All subjects gave their informed consent.

Blood sampling and biochemical determinations

All venous blood samples for the MORGEN study were collected in sitting position between 9.00 a.m. and 2 p.m. with no requirements for fasting. Information on the time of the last meal or beverage was recorded. The samples used for the preparation of plasma were collected in vacutainers containing 7.5% tri potassium EDTA (Safety-Monovette tubes, Sarstedt, Tilburg, the Netherlands) and centrifuged at room temperature for 10 minutes at 3000 x g. All samples for the main study were centrifuged within 1 h. After centrifugation the plasma was separated from blood cells, and stored at -20°C or -80°C until the homocysteine determination.

Blood collection for the stability study was accomplished in the same manner as in the MORGEN study; however, the samples were handled differently after drawing. Of each subject 2 vacutainers were collected with one venapuncture. One sample (2.6 ml) was centrifuged immediately at room temperature to obtain the baseline sample and one (9 ml) was stored at room temperature, which during the study varied between 18.2 and 21.8°C.

Each 15 minutes after centrifuging of the baseline sample (*T*0), we aspired 1.5 ml of whole blood out of the 9 ml tube and centrifuged it to obtain plasma to monitor the change in tHcy concentration after 15, 30, 45 and 60 minutes. We also measured the change at 75 and 90

minutes to enable comparisons with other stability studies, which did not report on the change within 1 h (9-11,16). The plasma samples of each subject were left at room temperature until all samples (*T*0 - *T*90) were acquired. Subsequently the plasma samples were stored at -20°C until the tHcy determination. Samples of each subject were batched together to be analyzed in the same run, encoded in such a manner that the laboratory technician was unaware of the processing time.

The biochemical analyses in Nijmegen were done with an automated high-performance liquid chromatography with reverse phase separation and fluorescent detection (Gilson 232-401 sample processor (Gilson Medical Electronics Inc., Middleton, WI), Spectra-Physics 8800 solvent delivery system and Spectra-Physics LC 304 fluorometer (San Jose, CA)), as described by Fiskerstrand (17), with some modifications (18). The within- and between-run coefficients of variation (CV) for reference plasma with this method were 3.3% and 8.9%, respectively. The samples measured in Bergen were analyzed with essentially the same method (17). The within- and between- day CV of this determination were reported to be <5% (17).

Statistical analysis

In the stability study the change in mean tHcy concentration was calculated by subtracting the tHcy concentration at baseline from the value at *T*15 to *T*90. Evaluation of a significant change was performed with the signed rank test. The ranking of subjects according to their baseline tHcy concentration and their tHcy concentration at *T*15 to *T*90 was assessed with the Spearman rank correlation. The mean change in tHcy concentration per minute was estimated using linear regression analysis with mean change in tHcy concentration at *T*15 to *T*90 as the dependent variable and time as the independent variable. The intercept of this regression line was set at 0, as at baseline there can neither be an increase nor a decrease in the tHcy concentration.

In the comparative study (and for the reproduction of these results) we used the tHcy concentration as measured in Bergen to predict the tHcy concentration as measured in Nijmegen by means of linear regression. The difference in mean tHcy concentration was assessed with the signed rank test. The Spearman rank correlation was used to compare the ranking of subjects according to their tHcy concentration as measured in Nijmegen and in Bergen.

Linear regression analyses with the data from the main study were performed after logarithmic transformation of tHcy levels, as this normalized the distribution. When logarithmic transformations were applied, geometric means are given (antilogarithms of the transformed means). The effect of the menopause was studied in women aged 45-54 years, as the number of pre-menopausal (n=183) and post-menopausal (n=175) women was almost equal in this group. The age-adjusted mean geometric tHcy concentration of pre- and post-menopausal

women was calculated and tested for differences by means of analysis of covariance. With the results of the stability study and the information on storage time at room temperature (in minutes) of the samples of our main study, we estimated what the mean tHcy concentration would have been if the samples were centrifuged immediately after drawing (i.e. the corrected value). In addition, we calculated the Spearman rank correlation coefficient between the measured and the corrected value. Finally, we compared our data with data of the Bergen laboratory using the results of the comparative study.

Findings were considered statistically significant if the two-sided P value was <0.05. We used the SAS statistical software (version 6.12) for all statistical analyses (SAS institute Inc., Cary, North Carolina, USA).

Results

Stability study: the effect of blood sampling conditions on the tHcy concentration

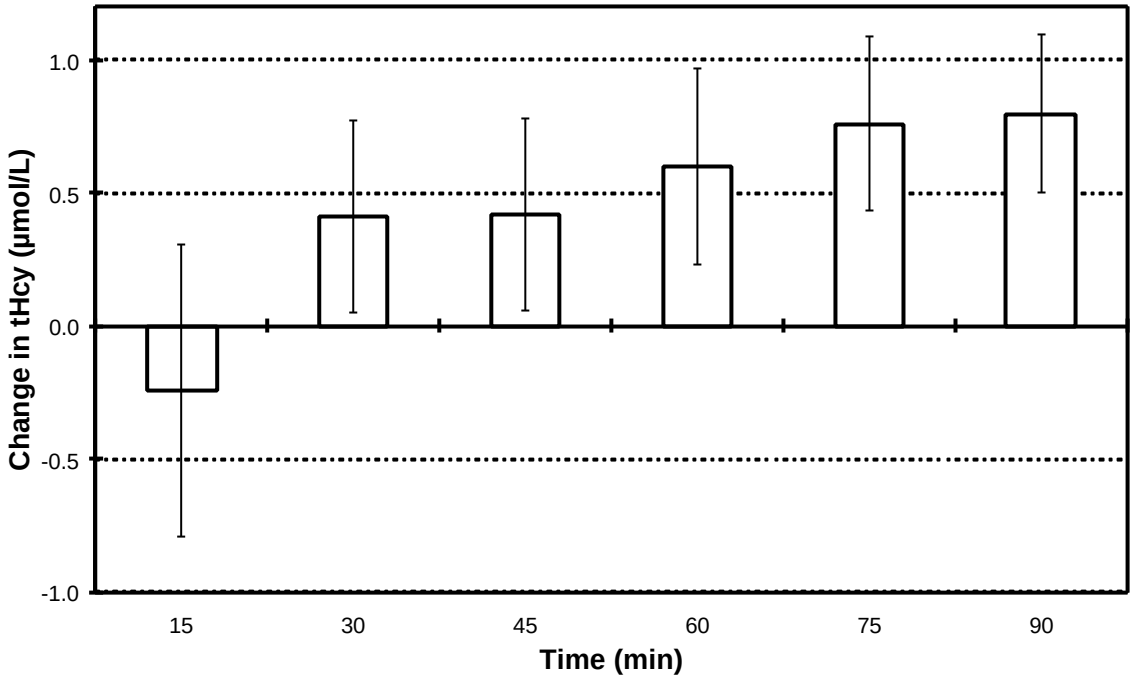
Figure 1 shows the time-dependent change in tHcy concentration from baseline, in whole blood stored at room temperature. Already 30 minutes after baseline, we observed a significant mean increase in tHcy concentration of 0.41 µmol/L (P=0.04). After 90 minutes the increase in tHcy concentration was on average 0.80 µmol/L (P=0.0001).

The correlation between the tHcy concentration at baseline and at *T*15, *T*30, *T*45, *T*60, *T*75 and *T*90 ranged between 0.95 and 0.97. This indicates that the ranking of the 26 subjects according to their tHcy concentration at baseline, was essentially the same at *T*15 to *T*90.

Comparative study: interlaboratory differences in the tHcy measurement

The mean tHcy concentration of the 88 samples was 15.4 µmol/L in Nijmegen and 13.2 µmol/L in Bergen (P=0.0001). The difference in tHcy concentration was systematic: predicting the tHcy level of Nijmegen with the tHcy level of Bergen with linear regression revealed an intercept of 2.4 µmol/L and a beta of 0.98. The correlation between the 2 measurements was 0.93, indicating that ranking subjects according to their tHcy concentration as measured in Nijmegen is in accordance with the ranking of the subjects in Bergen. These results were reproduced with an entirely different population (n=213) (15): the mean tHcy concentration was 15.0 µmol/L in Nijmegen and 12.7 µmol/L in Bergen (P=0.0001). The regression equation revealed a beta of 1.003 and an intercept of 2.2 µmol/L. The correlation between the 2 measurements was 0.86 indicating a similar ranking of the subjects according to the two laboratories. In figure 2 the Nijmegen and Bergen tHcy values are plotted against each other.

Figure 1 The time dependent mean change in tHcy concentration in whole blood stored at room temperature



The figure is based on data of 26 subjects (14 men and 12 women). Bars indicate mean absolute change (and 95% confidence intervals).

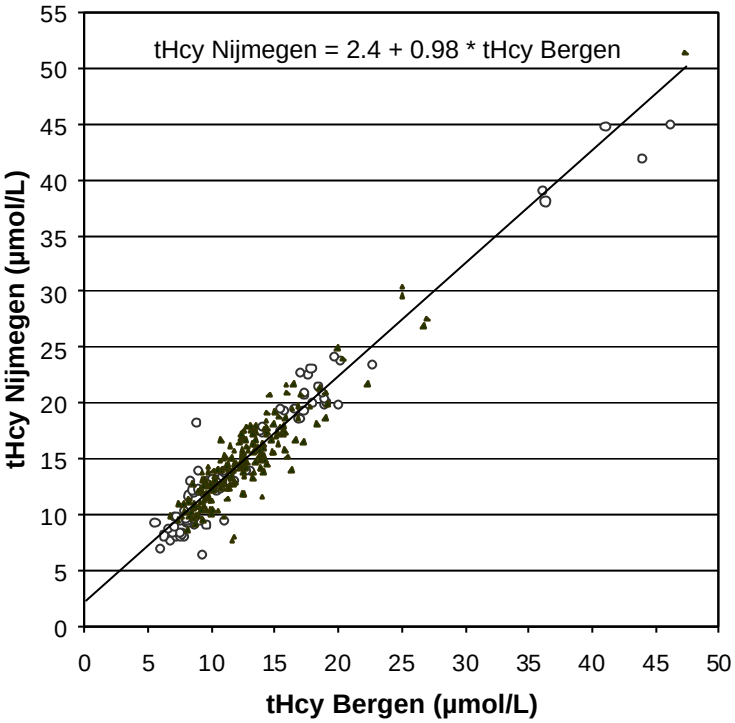
Main study: tHcy distribution in the general Dutch population

Figure 3 shows the positively skewed cumulative frequency distribution of the tHcy concentration for men and women separately. Subjects with a tHcy concentration >30 µmol/L (33 (2%) men and 14 (0.9%) women) are not shown in this curve. The arithmetic mean for all men was 14.6 (range 5.9-94.6) µmol/L and for all women 13.1 (6.1-72.2) µmol/L.

The geometric mean (and median) tHcy concentration by age and sex is given in table 1. For all age categories the tHcy level was higher in men than in women. In both sexes we observed an age-related increase in the tHcy concentration, which was statistically significant in women, but not in men.

In women, the largest increase in mean tHcy concentration was observed at the transition from the age category 40-49 to 50-59 years. To evaluate whether this effect was due to the occurrence of the menopause, we studied the separate effect of menopause in women aged 45-54 years. Within this group, menopausal status was not a significant predictor of the tHcy concentration. Furthermore, the age-adjusted mean tHcy concentration did not differ between pre and post-menopausal women (geometric means: 12.7 vs. 12.9 µmol/L, P=0.4).

Figure 2 Relation between tHcy concentration as measured in Nijmegen compared with the tHcy concentration as measured in Bergen



Explanation of symbols:

The circles are the values of 88 subjects participating in our main study. The tHcy concentration for these subjects was measured first in Nijmegen and re-measured in Bergen (comparative study).

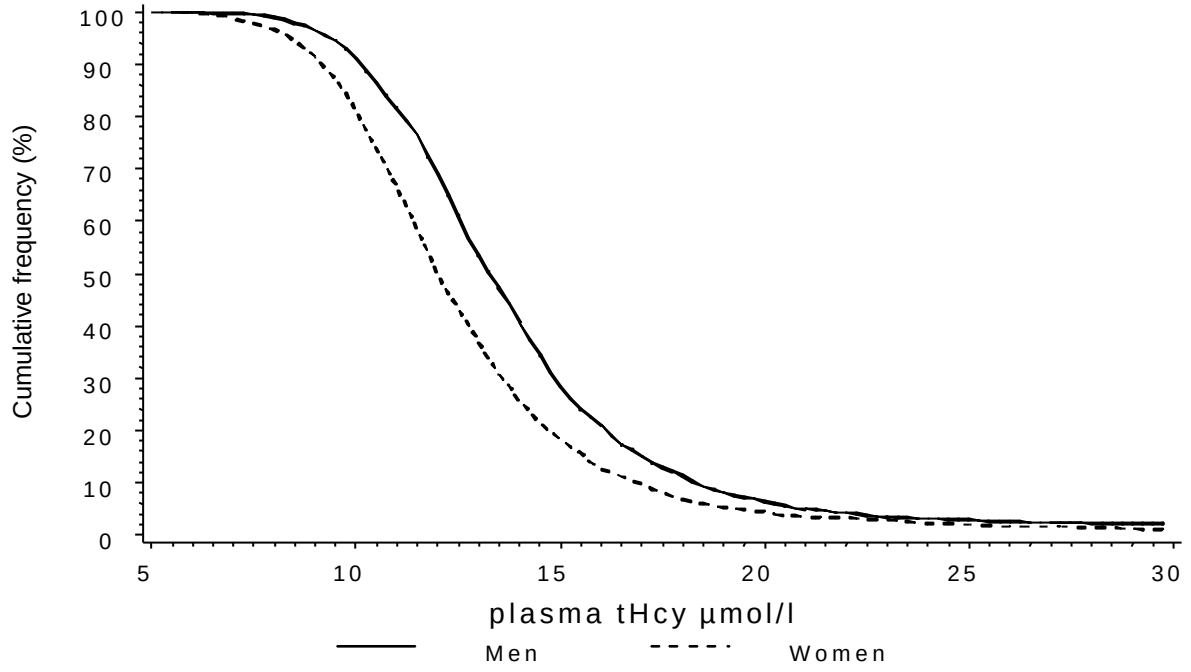
The triangles are the values of 213 subjects described in an earlier study (15). The tHcy concentration for these subjects was measured first in Bergen and re-measured in Nijmegen.

Combined results of the main study and the stability study

Linear regression analyses with data of the stability study predicted an increase in tHcy concentration of 0.0094 µmol/L per minute. With the information on storage time (in minutes) at room temperature of the samples of our main study, we could estimate the artificial increase in tHcy concentration for each sample, and correct for it. The corrected mean geometric tHcy level for men in the main study was 13.5 µmol/L compared with 13.9 µmol/L uncorrected (arithmetic: 14.3 vs. 14.6 µmol/L) and for women 12.2 µmol/L compared with 12.6 µmol/L uncorrected (arithmetic: 12.8 vs. 13.1 µmol/L).

Storage time at room temperature varied between the samples (0-60 minutes, with a mean time of 36 minutes), which resulted in differences in increases. However, the correlation between the corrected and measured tHcy concentration was 0.99 for both men and women, indicating that the artificial increase had not affected the ranking of subjects.

Figure 3 Cumulative frequency distribution of the tHcy concentration in Dutch men and women aged 20-65 years



Combined results of the main study and the comparative study

As the systematic difference between the tHcy concentration of Nijmegen and Bergen was -2.4 µmol/L, we adapted the definition of hyperhomocysteinemia from Bergen (i.e. tHcy >15 µmol/L (19)) to 17.4 µmol/L. The prevalence of hyperhomocysteinemia in our main study with the adapted definition was 14.4% in men and 8.9% in women. With the >15 µmol/L definition of hyperhomocysteinemia, 30.9% of the men and 19.9% of the women would have been diagnosed as hyperhomocysteinemic.

Table 1 Mean (median) geometric tHcy (and 95% confidence interval) concentration in µmol/L in the main study by age and sex

	Men		Women	
Age	N	tHcy	N	tHcy
20-65 y	1493	13.9 (13.5) (13.7-14.1)	1532	12.6 (12.2) (12.4-12.8)[a]
20-29 y	359	13.7 (13.1) (13.3-14.1)	358	12.2 (11.8) (11.8-12.5)[a]
30-39 y	360	13.9 (13.3) (13.5-14.3)	360	12.5 (12.3) (12.2-12.9)[a]
40-49 y	360	13.7 (13.4) (13.3-14.1)	358	12.4 (12.1) (12.0-12.7)[a]
50-59 y	358	14.2 (13.9) (13.7-14.6)	360	13.1 (12.9) (12.7-13.4)[a]
60-65 y	56	14.6 (14.3) (13.6-15.7)	96	13.5 (13.0) (12.8-14.3)
P for trend by age		0.1		0.0001

Explanation of symbols: a=Significantly different from men in same age category, P=0.0001.

Table 2 shows the non-fasting tHcy percentiles of our Dutch study population compared with the fasting tHcy percentiles of the control group of the European Concerted Action Project on vascular disease (COMAC) (20). The controls of this project originate from 9 European countries. As the tHcy measurements for the COMAC study were centrally performed in Bergen, we subtracted 2.4 µmol/L of our original percentile values, in order to make a valuable comparison. The comparison shows that the tHcy values in this Dutch study were higher in younger as well as older men and women, compared with the values of the COMAC study. Furthermore, at higher percentiles the difference between the Dutch and the COMAC concentrations became larger. For example, in women younger than 45 years, the difference was 0.4 µmol/L at the 2.5th percentile, 1.6 µmol/L at the 50th percentile and 5.3 at the 97.5th percentile.

Table 2 The non-fasting tHcy concentration in Dutch adults compared with the fasting tHcy concentration in the control group of the European Concerted Action Project on homocysteine and vascular disease (COMAC study)

	Percentile[a]				
	2.5	20	50	80	97.5
Dutch study, all (n=3025)	6.0	8.4	10.5	13.3	22.2
COMAC, all (n=800)	5.9	7.7	9.5	12.1	18.5
Dutch men <45 years (n=899)	6.3	8.7	10.9	14.0	27.5
COMAC men <45 years (n=232)	6.3	7.8	9.6	11.9	18.8
Dutch women <45 years (n=898)	5.2	7.7	9.7	12.2	22.2
COMAC women <45 years (n=135)	4.8	6.6	8.1	10.4	16.9
Dutch men ≥45 years (n=594)	7.1	9.4	11.3	14.0	20.3
COMAC men ≥45 years (n=338)	6.8	8.5	10.3	12.6	18.6
Dutch women ≥45 years (n=634)	6.2	8.3	10.1	13.1	20.9
COMAC women ≥45 years (n=95)	5.8	7.1	9.1	11.4	15.1

Explanation of symbols: a=To make a comparison with the COMAC study (20) possible, we subtracted 2.4 µmol/L of our original percentile values.

Discussion

The goal of our study was to provide data on the tHcy distribution in the Dutch adult population. Two problems that could hinder a correct judgement of the tHcy status were evaluated in the stability and the comparative study. The tHcy concentration was skewed towards higher values, the geometric mean tHcy level was higher (13.9 µmol/L) in men than in women (12.6 µmol/L) and increased with age. The stability study indicated that our sampling conditions might have caused a mean artificial increase of 0.4 µmol/L in tHcy concentration, for both sexess. The comparative study showed a systematic difference of 2.4 µmol/L between the

laboratory in Nijmegen and Bergen. Taking this difference into account revealed a higher tHcy concentration in the Netherlands than in other European countries.

Factors that might have contributed to random and systematic errors in our data will now be discussed. First, it is generally recommended that the subjects should be fasting for the tHcy measurement (21). Consumption of a normal breakfast is associated with a lower tHcy concentration (9) and a protein rich meal result in a higher tHcy concentration up until 8 h after consumption (22). Our data showed no significant difference in tHcy concentration in subjects who had fasted before the blood collection (n=89), compared with subjects who where non-fasting (geometric means in men: 14.5 vs. 13.9 µmol/L, P=0.4; in women: 13.3 vs. 12.6 µmol/L, P=0.1).

Second, the tHcy concentration in samples stored at -20°C remains stable over at least 2 years (9,10,23,24). Our samples were stored at this temperature for a median period of 4 (range 0-17) months, while the major part of the total storage time was at -80°C (median 30, range 11-51 months). As the stability at -80°C is even better than at -20°C, frozen storage time will not have affected the tHcy concentration.

Third, the intra-individual variation in the tHcy concentration is ~9% over a year, with little seasonal variation (25-28), resulting in reliability coefficients (the between person variance as a proportion of the total variance) between 0.82 and 0.90 (26-28). Therefore, a single measurement characterizes a person with respect to his/her tHcy concentration reasonably well.

Fourth, all samples of the main study were measured between July 1997 until January 1999. Over this period the within- (repeatability) and between-run (reproducibility) coefficients of variation for reference plasma (≥2 samples in each run) with this method were respectively 3.2% and 8.6%, indicating that the precision of the method is good. The extent of a systematic error is difficult to establish for the tHcy analysis, as there is no gold standard. Nevertheless, Fiskerstrand *et al* (17), who used essentially the same method as we did, observed that known concentrations of homocysteine added to water or to plasma showed an average recovery of 95%, demonstrating that the validity of this method is appropriate.

Finally, our study population consisted of a random sample from the MORGEN study. All subjects invited for the MORGEN study received a response card enclosed in their invitation letter. This card was returned by ~70%, and ~50% completed the full assessment. A non-response study indicated that non-responders were more likely to be men and to be smokers. As men and smokers, on average, have a higher tHcy concentration (29), this bias will influence our tHcy distribution into the direction of lower values. However, the percentage of males (49%) and smokers (36%) in our study was similar as in a nationwide study (30). Furthermore, other characteristics of our study population (education and employment) were also comparable to nationwide data (30). Therefore, we conclude that selective response most likely did not largely influence our results.

Taken together, all factors evaluated above do not appear to have seriously affected the internal validity and precision of our estimate of the tHcy distribution in the Dutch population. However, our blood sampling conditions introduced a bias due to a continuous synthesis and transport of homocysteine in blood cells at room temperature (11,31). The stability study indicated that the MORGEN sampling protocol increased the tHcy concentration with 0.6 µmol/L per h, which is in line with other stability studies (9-11,16). The increase was independent of the baseline tHcy concentration and of the amount of blood cells (data not shown). Estimating the increase for each subject and subtracting that from the measured concentration showed that the corrected mean concentration might have been 0.4 µmol/L lower. Fortunately, the increase did neither affect the ranking of the subjects according to their tHcy concentrations nor appreciably change the prevalence of hyperhomocysteinemia.

The tHcy status of our population was evaluated by comparing our data with those from other populations. The largest problem in this evaluation, apart from differences in ethnicity, dietary habits, genotype distribution, etc., is the large interlaboratory variability in the tHcy measurement. For example, the coefficient of variation of 1 sample sent to 5 laboratories was 15% (13) and 9% for 9 laboratories (12). Therefore, data derived from different laboratories cannot be compared unless the interlaboratory differences are known.

The comparative study, and the reproduction of these results in an entirely independent population (15), indicated a systematic difference of -2.4 µmol/L in the measured tHcy concentration between the laboratory in Bergen and our laboratory. This difference cannot be attributed to differences in the sampling conditions, as they were exactly the same. The only difference was that the samples that were sent to Bergen had already been thawed twice for other biochemical measurements, whereas the vast majority of the samples measured in Nijmegen were thawed for the first time when they were analyzed. This, however, should not have influenced the results as repeated freezing and thawing cycles do not affect the tHcy concentration (19). Therefore, the only plausible explanation for the systematic difference is a difference in the calibration of the tHcy concentration.

Comparing the adapted non-fasting tHcy percentiles of our study with fasting percentiles from the controls of the COMAC study (20) (table 2), and with non-fasting tHcy levels (determined in Bergen) of the population-based Hordaland Homocysteine Study in Norway (32), showed that the tHcy concentrations in our study were higher and more skewed towards higher values. The difference between the Dutch and European values may in theory even be larger, as fasting levels (COMAC study) might be higher than non-fasting levels (9). In this evaluation we assume a negligible effect of the Dutch (3%) (15) and hospital based (2%) (4) controls that were included in the COMAC study.

In accordance with the Hordaland Homocysteine Study (32), we observed a higher tHcy concentration in older subjects. In women, we did not observe an effect of the menopause on

top of the age related increase. Despite the evidence that the response in tHcy concentration after a methionine load is larger in post-menopausal women compared with pre-menopausal women (33,34), the lack of a significant effect of the menopause on the fasting tHcy concentration has been observed before (35,36).

In conclusion, this study provides precise and valid data of the tHcy status in the Dutch population aged 20-65 years. We plead for an international standardization or calibration of the tHcy measurement by an external quality assurance program. This will improve the quality of the tHcy measurement and will enable comparisons of tHcy data between studies, resulting in better estimates of the prevalence of hyperhomocysteinemia. For example, if we had used 15 µmol/L as a cut-off level (19), the prevalence of hyperhomocysteinemia in the Netherlands would have been twice as high. The correct use of the cut-off level decreased the number of hyperhomocysteinemic subjects, nevertheless, the average tHcy concentration in this population is worse than in other European countries. Future studies must provide knowledge into factors that cause this unfavorable tHcy concentration in order to develop optimal public health strategies to lower the tHcy concentration in the general Dutch population and thereby possibly reducing the risk for the development of CVD.

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4

Dietary intake of B-vitamins and plasma homocysteine in the general Dutch adult population

Background: An elevated plasma total homocysteine (tHcy) concentration is associated with an increased risk of cardiovascular diseases. Folate, vitamin B2, B6 and B12 are essential in homocysteine metabolism.

Objective: To describe the association between dietary intakes of folate, riboflavin (vitamin B2), vitamin B6, and vitamin B12 and the non-fasting plasma tHcy concentration.

Design: A random sample of 2435 men and women aged 20-65 years, from a population- based Dutch cohort were analyzed cross-sectionally.

Results: Univariately, intakes of all B-vitamins were inversely related to the plasma tHcy concentration. In multivariate models, only folate intake remained inversely associated with the plasma tHcy concentration. Mean plasma tHcy concentrations (adjusted for intakes of riboflavin, vitamin B6, vitamin B12, and methionine and for age, smoking, and alcohol consumption) in men with low (first quintile: 161 µg/d) and high (fifth quintile: 254 µg/d) folate intakes were 15.4 and 13.2 µmol/L, respectively; in women plasma tHcy concentrations were 13.7 and 12.4 µmol/L at folate intakes of 160 and 262 µg/d, respectively. In men, the difference in the mean plasma tHcy concentration between men with low and high folate intakes was greater in smokers than in non-smokers (2.8 compared with 1.6 µmol/L), and greater in non-drinkers than in drinkers of >2 alcoholic drinks/d (3.5 compared with 1.4 µmol/L). In women, the association between folate intake and plasma tHcy concentration was not modified by smoking or alcohol consumption.

Conclusions: In this Dutch population, folate was the only B-vitamin independently inversely associated with the plasma tHcy concentration. Changing dietary habits may substantially influence the plasma tHcy concentration in the general population.

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Introduction

The relation between elevated plasma homocysteine concentrations and cardiovascular diseases has been suspected since the 1960s. Extremely high plasma homocysteine concentrations in patients with homocystinuria, a rare genetic disorder of impaired homocysteine metabolism, are associated with the development of atherosclerosis (1,2). More recently, numerous studies showed that a moderate elevation of the plasma total homocysteine (tHcy) concentration is also associated with an increased risk of vascular diseases (3,4). Although the exact mechanism underlying this association is unidentified, it is suggested that homocysteine or one of its metabolites contributes to the development of vascular diseases (5-8).

Homocysteine is a sulfur-containing amino acid derived from the metabolism of the essential amino acid methionine, which is the only dietary precursor of homocysteine. Several B-vitamins are involved in homocysteine metabolism. Vitamin B6 is the cofactor of cystathionine β-synthase, the enzyme that irreversibly converts homocysteine to cystathionine. Vitamin B12 is the cofactor of methionine synthase, which remethylates homocysteine to methionine. Folate, in the 5-methyltetrahydrofolate form, donates the methyl-group in this reaction. For one of the recycling steps to reform 5-methyltetrahydrofolate, the enzyme methylenetetrahydrofolate reductase is necessary. This enzyme, in turn, uses riboflavin (vitamin B2) as a cofactor (9,10).

Several intervention studies have provided evidence for the importance of B-vitamins in homocysteine metabolism. Supplements containing folic acid (synthetic form of folate) and also combinations of folic acid, riboflavin, vitamin B6 and B12 effectively reduced the plasma tHcy concentrations in subjects with normal (11,12) and elevated (13-16) baseline concentrations.

Although information on the relation between dietary intakes of folate, riboflavin, vitamin B6 and B12, and the plasma tHcy concentration is available for middle aged (17) and elderly (18,19) populations, it is scarce for the general adult population. This information is essential for an understanding of to what extent the plasma tHcy concentration can be lowered in the general population through dietary interventions. The only available large population-based study of adults (40-67 years), the Hordaland Homocysteine Study, unfortunately did not provide this information because it lacked detailed information on dietary intakes (20). The present study contains detailed information on intakes of folate, riboflavin, vitamin B6 and B12 and describes the association between these vitamins and plasma tHcy concentrations in a random sample (n=2435) of the general Dutch population aged 20-65 years.

Subjects and methods

Study population

Data were used from the MORGEN study (21), a cross-sectional investigation into the prevalence of risk factors for chronic diseases and certain chronic conditions. The population of this study consisted of a random sample of subjects aged 20-65 years from 3 cities in the Netherlands: Amsterdam, Doetinchem, and Maastricht. The external Medical-Ethical Committee of the TNO Toxicology and Nutrition Institute, which follows the guidelines of the Helsinki Declaration, approved the protocol of the MORGEN study.

Of the 19,066 subjects who participated in the MORGEN study during 1993-1996, 14,356 were considered eligible for the present study because they had completed questionnaires, had blood available for all aimed biochemical analyses, and had recorded time of drawing and centrifugation of blood. Of those subjects, 7992 subjects were excluded because their whole blood samples had been stored at room temperature for >1 h, which may have artificially increased the plasma tHcy concentration (22,23). A sample of 6364 subjects remained, from which an age- and sex- stratified random sample of 3025 subjects was drawn. Demographic variables and cardiovascular disease risk factors of this sample did not differ significantly from those of the MORGEN study (1993-1996). Furthermore, the distribution of demographic characteristics (e.g., sex, employment, and education) of our sample was comparable with nationwide data (23).

Data collection

Respondents answered 2 self-administered questionnaires: a general and a semi-quantitative food-frequency questionnaire. Subsequently, trained research assistants performed a physical examination of each subject at the Municipal Health Service.

From the general questionnaire we extracted information on sex, age, smoking and alcohol consumption. The food-frequency questionnaire assessed the habitual consumption of 178 food items and vitamin supplements during the previous year. The frequency of consumption of main items could be indicated in 1) times per day, 2) times per week, 3) times per month, 4) times per year or 5) never. The amount eaten was estimated in commonly used units by using household measures or colored photographs of foods showing different portion sizes (24).

Average daily intakes of folate, riboflavin vitamin B6, B12, methionine and total energy were estimated by multiplying the frequency of consumption of the food items by the portion size and the nutrient content per gram. Riboflavin, vitamin B6 and energy intakes were calculated by using an extended version of the 1996 computerized Dutch food composition table (25). This table does not contain data on folate, vitamin B12 or methionine. Therefore, folate data were derived from a validated HPLC method with which Dutch foods were analyzed (26,27). Vitamin

B12 and methionine data were compiled from food composition tables from the United States, United Kingdom, Germany, and Sweden.

The food-frequency questionnaire did not collect information on the doses and contents of vitamin supplements. Hence, we excluded from all analyses subjects who used supplements that might have contained B-vitamins (217 men and 371 women), to reduce misclassification of B-vitamin intake. In addition, one man and one woman were excluded because of unreliable data in the food-frequency questionnaire.

Blood sampling and biochemical determinations

During the physical examination non-fasting venous blood samples were obtained from subjects in a sitting position. The samples used for the preparation of plasma were collected in evacuated tubes containing vacutainers containing 7.5% tripotassium EDTA (Safety-Monovette tubes, Sarstedt, Tilburg, Netherlands) and were centrifuged at room temperature for 10 minutes at 3000 x *g* within 1 h of collection. After centrifugation the plasma was separated from blood cells immediately and stored at -20°C or -80°C until homocysteine was measured.

The plasma tHcy concentration, which includes both the unbound and bound fractions of homocysteine, was measured by HPLC with fluorescence detection as described by Fiskerstrand *et al* (28), with some modifications (29). All samples were measured between July 1997 and January 1999; the within- and between-run CVs for reference plasma (≥2 samples in each run) were 3.2% and 8.6%, respectively.

Statistical analysis

Because users of B-vitamin supplements were excluded, all analyses were based on the data of 1275 men and 1160 women. Differences in characteristics between men and women were tested with Wilcoxon's two-sample test. The distributions of nutrient and energy intakes, and plasma tHcy concentrations were positively skewed; therefore, we used natural logarithmic transformations to normalize their distributions. Thus, geometric means (antilogarithms of the transformed means) are presented unless stated otherwise.

To assess the association between intake of B-vitamins and the plasma tHcy concentration, the nutrients (B-vitamins and methionine) were energy-adjusted by regressing nutrient intake on total energy intake for each individual. The energy independent residuals of this analysis were standardized to the predicted nutrient intake at the average energy intake (9391 kJ/d) in our population (30). Energy adjustments were applied to prevent multicollinearity in the multivariate models. The adjustments reduced the Spearman correlation between the nutrients from 0.46-0.81 to 0.12-0.69 and resulted in a maximum variance inflation factor (VIF) of 2.7. Multicollinearity is a concern when the VIF is larger than 10.0 (31).

Sex-specific quintiles of B-vitamin intake were created after the logarithmic transformations and energy adjustments. Each relation between B-vitamin and plasma tHcy was evaluated by calculating the mean plasma tHcy concentration per quintile of B-vitamin intake. Adjusted mean plasma tHcy concentrations were estimated by analysis of covariance. Adjustments were made for age (y), methionine intake (mg/d), smoking (no or yes), and alcohol consumption. Alcohol consumption was included as 2 indicator variables: ≤2 drinks/d and >2 drinks/d, with non-drinkers with as a reference. Non-drinkers comprised abstainers (128 men and 273 women) and those drinking <1 drink/wk (209 men and 397 women). These variables were associated with B-vitamin intake and with the plasma tHcy concentration. We also adjusted for the intake of the 3 other B-vitamins under study (included continuously in the model when considered as confounders) to assess the independent effect of each B-vitamin on the plasma tHcy concentration.

Trends across the quintiles were evaluated with linear regression, in which the quintiles were modeled as continuous variables. For the multivariate models similar adjustments were made as in the analyses of covariance. The regression coefficients of these analyses express a proportional change, because the plasma tHcy concentration was logarithmically transformed.

The relation between folate intake and the plasma tHcy concentration was visualized by plotting the adjusted mean plasma tHcy concentration against the mean folate intake in gender specific deciles of folate intake. The adjusted means were calculated in a manner similar to that described for the quintiles.

Because both smoking and alcohol consumption are known to interfere with B-vitamin metabolism (32-35), they were also evaluated as effect modifiers. For this evaluation the P value of the interaction terms (e.g. smoking × folate intake) was assessed for significance in the multivariate linear regression models.

Differences in the mean plasma tHcy concentration compared with a referent category were tested by using Bonferroni's adjustment for multiple comparisons. Findings were considered statistically significant if the two-sided P value was <0.05. We used the SAS statistical software (version 6.12) for all statistical analyses (SAS Institute Inc., Cary, NC).

Results

Population characteristics

Selected characteristics of the study population are shown in table 1. Because B-vitamin supplements are used more frequently by women than by men, the exclusion of B-vitamin supplement users resulted in a greater number of men in the study. However, the age range of the men and women was similar. The plasma tHcy concentration was significantly higher in

men than in women, as were B-vitamin and methionine intakes, and alcohol consumption. The percentage of smokers was similar between the sexes.

Table 1 Selected characteristics of the study population

Characteristics	Men (n=1275)		Women (n=1160)	
	Mean	SD	Mean	SD
Age (years)	40.5	12.1	40.8	12.5
tHcy (µmol/L)[a]	14.8	6.2	13.4[b]	4.8
Folate intake (µg/d)[a]	239	73	192[b]	54
Vitamin B2 (Riboflavin) (mg/d)[a]	1.8	0.7	1.5[b]	0.5
Vitamin B6 (mg/d)[a]	2.1	0.6	1.6[b]	0.4
Vitamin B12 (µg/d)[a]	5.7	2.8	4.3[b]	2.1
Methionine (mg/d)[a]	2.1	0.6	1.7[b]	0.5
Alcohol (drinks/d)	1.7	2.2	0.5[b]	1.0
Current smokers (%)	35.1		36.5	

Explanation of symbols: a=Arithmetic values, b=Significantly different from men, P=0.0001.

Association between B-vitamin intake and the plasma tHcy concentration

Mean plasma tHcy concentrations by quintiles of B-vitamin intake are shown in table 2. Univariately, the intake of all B-vitamins was inversely associated with the plasma tHcy concentration in both men and women. An increase in intake of the different B-vitamins from the lowest (first) to the highest (fifth) quintile was associated with decreases in plasma tHcy concentration of 0.7 to 2.8 µmol/L. The largest decrease was observed across quintiles of folate intake; plasma tHcy concentration decreased from 15.8 to 13.0 µmol/L in men and from 14.2 to 12.2 µmol/L in women.

Multivariate regression analyses showed that, after adjustment for age, intake of the other B-vitamins and methionine, smoking and alcohol consumption, only folate intake remained inversely associated with the plasma tHcy concentration (table 2). The mean plasma tHcy concentration decreased from 15.4 µmol/L in the first quintile of folate intake to 13.2 µmol/L in the fifth quintile in men and from 13.7 to 12.4 µmol/L in women. Each quintile increase in folate intake was associated with a 3.2% decrease in plasma tHcy in men and a 2.5% decrease in women.

The association between folate intake and the plasma tHcy concentration in both sexes described a curve, especially in women, with a steeper slope in the low folate intake range (140-210 µg/d), which gradually became flatter at higher folate intakes (figure 1). Across the range of folate intakes, the curve continued to describe an inverse association.

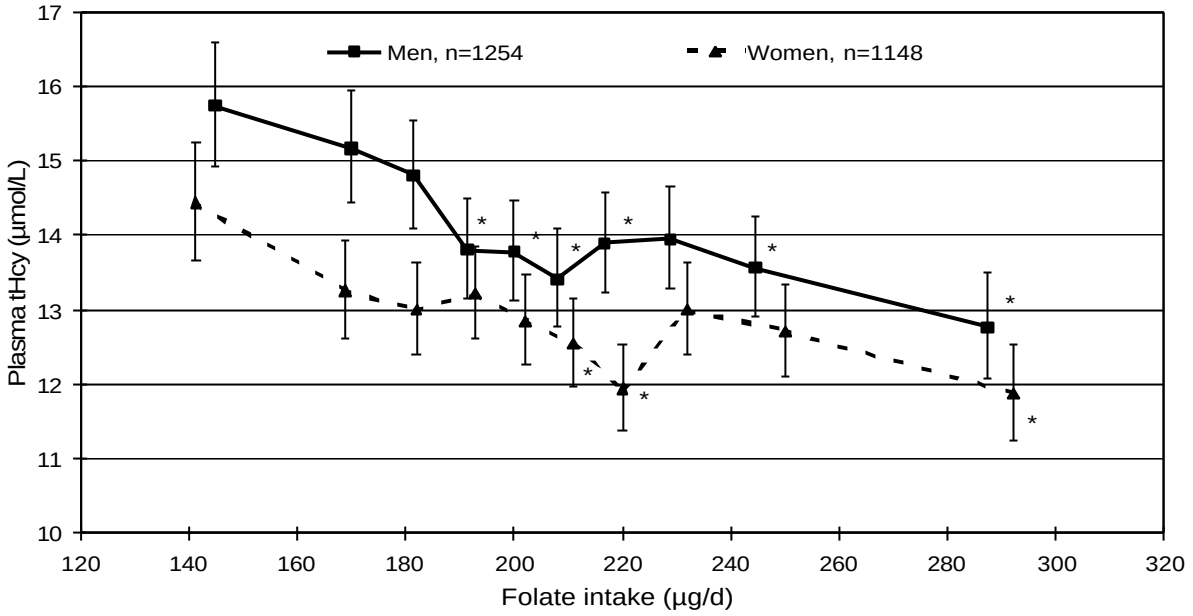
Table 2 Mean plasma total homocysteine (tHcy) concentrations by quintiles of B-vitamin intake

	Men (n=1275)[a]					
	Q1	Q2	Q3	Q4	Q5	P for trend
Folate (µg/d)[b]	161	187	204	223	254	
tHcy	15.8	14.4[e]	13.5[e]	13.8[e]	13.0[e]	<0.001
Adjusted[c] tHcy	15.4	14.3[d]	13.6[e]	13.9[e]	13.2[e]	<0.001
Vitamin B2 (mg/d)[b]	1.1	1.3	1.5	1.7	2.1	
tHcy	14.5	14.5	13.9	13.8	13.7	<0.01
Adjusted[c] tHcy	13.8	14.4	14.0	14.0	14.2	0.6
Vitamin B6 (mg/d)[b]	1.5	1.7	1.8	2.0	2.2	
tHcy	15.4	14.0[e]	13.9 [e]	13.8[e]	13.3[e]	<0.001
Adjusted[c] tHcy	14.6	13.9	13.9	14.1	13.8	0.2
Vitamin B12 (µg/d)[b]	2.9	3.9	4.6	5.5	7.2	
tHcy	14.7	14.5	14.2	13.5[e]	13.4[e]	<0.001
Adjusted[c] tHcy	14.0	14.3	14.1	13.6	14.3	0.8
	Women (n=1160)[a]					
	Q1	Q2	Q3	Q4	Q5	P for trend
Folate (µg/d)[b]	160	188	206	224	262	
tHcy	14.2	13.0[e]	12.6[e]	12.3[e]	12.2[e]	<0.001
Adjusted[c] tHcy	13.7	13.1	12.7[e]	12.5[e]	12.4[e]	<0.001
Vitamin B2 (mg/d)[b]	1.1	1.4	1.6	1.8	2.2	
tHcy	13.4	13.2	12.8	12.5[d]	12.5[d]	<0.001
Adjusted[c] tHcy	12.8	12.9	12.7	12.7	13.2	0.6
Vitamin B6 (mg/d)[b]	1.4	1.6	1.7	1.9	2.1	
tHcy	14.0	12.8[e]	13.0[d]	12.6[e]	12.1[e]	<0.001
Adjusted[c] tHcy	13.1	12.6	13.1	12.9	12.6	0.6
Vitamin B12 (µg/d)[b]	2.9	3.7	4.4	5.3	6.8	
tHcy	13.6	13.0	12.6[d]	12.5[d]	12.6[d]	<0.001
Adjusted[c] tHcy	12.8	12.8	12.6	12.7	13.4	0.3

Explanation of symbols: a=Due to missing values the adjusted analyses are based on 1254 men and 1148 women; b=Median geometric intake per quintile standardized to average energy intake in our population (i.e. 9391 KJ/d); c=Adjusted for intake of other B-vitamins, intake of methionine, age, smoking (no/yes) and alcohol consumption [drinkers (≤2 and >2 alcoholic drinks/d) vs. non-drinkers]; d and e=Significantly different compared from the lowest folate intake quintile: d=P<0.05, e=P<0.01.

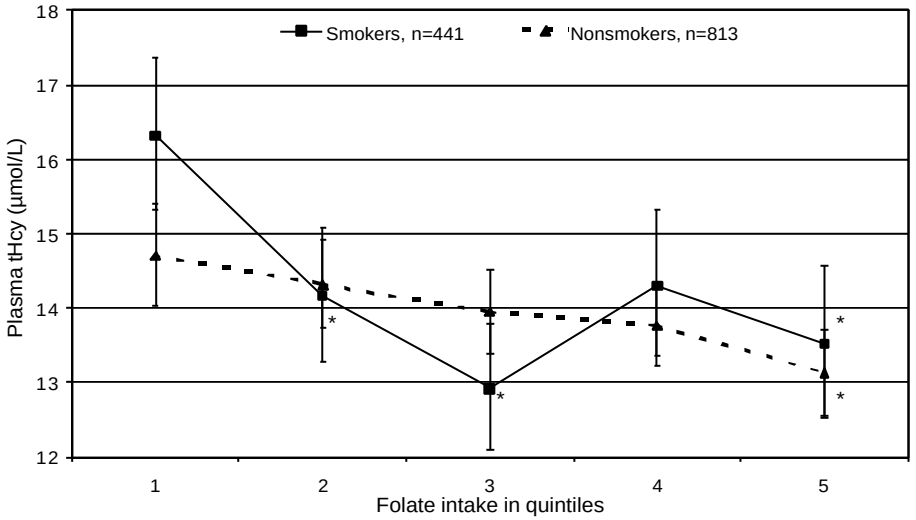
In men the relation between folate intake and the plasma tHcy concentration differed by smoking status (figure 2). An increase in folate intake of one quintile was associated with a 2.6% decrease in plasma tHcy in non-smokers (P for trend <0.01) and a 4.1% decrease in smokers (P for trend <0.001). In non-smokers the mean plasma tHcy concentration decreased with 1.6 µmol/L from the first to the fifth quintile, whereas in smokers it decreased 2.8 µmol/L. In women we observed that the relation between folate intake and the plasma tHcy concentration was also somewhat stronger in smokers than in non-smokers, though a statistically significant interaction was absent.

Figure 1 Mean plasma total homocysteine (tHcy) concentration in men and women by deciles of folate intake



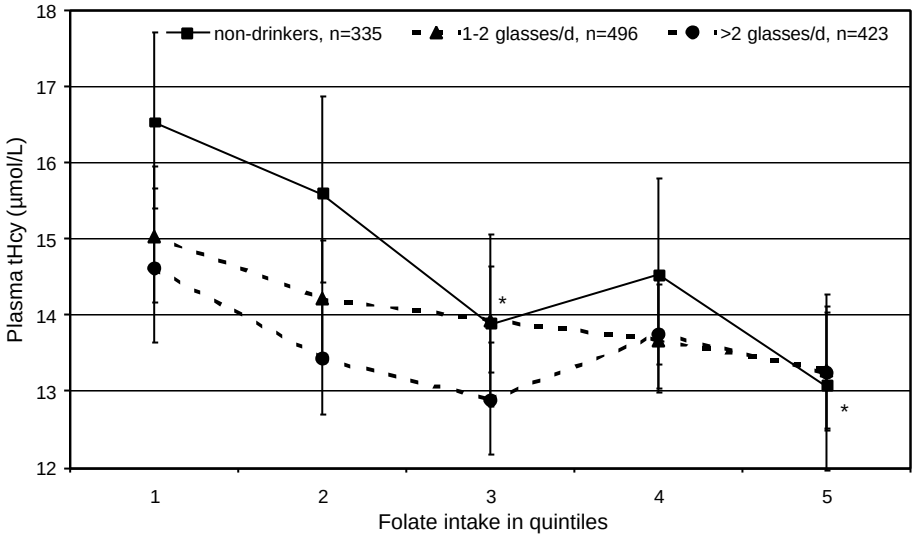
Explanation of symbols: Means were adjusted for intake of riboflavin, vitamin B6 and B12, methionine, age, smoking (no/yes) and consumption [drinkers (≤2 and >2 alcoholic drinks/d) vs. non-drinkers]. Folate intake was standardized to average energy intake in our population (i.e. 9391 KJ/d). Asterisks indicate significantly different from the lowest decile of folate intake, P<0.05. Bars represent 95% confidence intervals.

Figure 2 Mean plasma total homocysteine (tHcy) concentration by quintiles of folate intake in men, stratified for smoking



Explanation of symbols: Means were adjusted for intake of riboflavin, vitamin B6 and B12, methionine, age and alcohol consumption [drinkers (≤2 and >2 alcoholic drinks/d) vs. non-drinkers]. Asterisks indicate significantly different from the lowest quintile of folate intake, P<0.05. Bars represent 95% confidence intervals.

Figure 3 Mean plasma total homocysteine (tHcy) concentration by quintiles of folate intake in males, stratified for alcohol consumption



Explanations of symbols: Means are adjusted for intake of riboflavin, vitamin B6 and B12, methionine, age and smoking (no/yes). Asterisks indicate significantly different from the lowest quintile of folate intake, P<0.05. Bars represent 95% confidence intervals.

In men we observed that alcohol consumption also modified the relation between folate intake and the plasma tHcy concentration (figure 3). An increase of one quintile in folate intake was associated with a decrease in the plasma tHcy of 5.4% (P for trend <0.001) in non-drinkers, 2.8 % (P for trend <0.01) in drinkers of ≤2 drinks alcohol/d, and 1.5% (P for trend=0.2) in drinkers of >2 drinks alcohol/d. The highest plasma tHcy concentration was observed in non-drinkers with a low folate intake (first quintile: 16.5 µmol/L). In women, alcohol did not modify the relation between folate intake and the plasma tHcy concentration.

Discussion

This was the first study in which the association between dietary intake of the B-vitamins involved in homocysteine metabolism and the non-fasting plasma tHcy concentration was investigated for a large population-based sample aged 20-65 years. Our results indicate that after adjustment for confounders, folate was the only B-vitamin independently inversely associated with the plasma tHcy concentration. Furthermore, we showed that in men this relation was stronger in smokers and weaker in alcohol drinkers.

In an earlier study we showed that our measurement of the plasma tHcy concentrations is precise and valid, and thus suitable for use when studying associations (23). Habitual dietary intake data were collected with a semi-quantitative food-frequency questionnaire. A validation study indicated that the reproducibility and relative validity of food groups predominantly contributing to the intake of the B-vitamins (i.e. milk and milk products, meat, potatoes, bread

and vegetables) for this questionnaire were acceptable and comparable with those of other food-frequency questionnaires (24).

Our finding that folate is an important dietary correlate of the plasma tHcy concentration confirms the findings of observational studies in middle-aged (17) and elderly (18,19) subjects. In contrast with those studies, however, we observed no association between plasma tHcy and riboflavin (17), vitamin B6 (17-19) and B12 (17), probably because of the more detailed statistical adjustment that we applied. For example, using the same model as used in the Framingham study (18), we found an association between vitamin B6 and the plasma tHcy concentration in men only but not in our complete model. Alcohol consumption emerged as the strongest confounder in this association; methionine intake was the next strongest confounder. Moreover, the other 2 studies (17,19) did not correct the intake of one B-vitamin for the intake of the others. We suspect that if this had been done, there would have been no associations between the plasma tHcy concentration and riboflavin, vitamin B6, and B12. This suspicion is suggested by the weakening of these associations after correction for breakfast cereals, many of which are fortified with vitamins in the United States (17).

Because different B-vitamins are frequently present in the same foods, the intake of one vitamin can be a marker for the intake of another vitamin. Our results showed that the univariate associations between the plasma tHcy concentration and riboflavin, vitamin B6 and B12 existed mainly because of confounding by folate intake. The importance of folate can be explained by its role in homocysteine metabolism. Folate, in the 5-methyltetrahydrofolate form, is the substrate donor in the remethylation of homocysteine to methionine (9). In contrast, the other B-vitamins are cofactors of enzymes and are not used up during homocysteine degradation; thus, they are not often a limiting factor.

The cross-sectional design of our study limited our identification of causal relations; however, recent intervention trials showed that increases in the dietary folate intake resulted in decreases in plasma tHcy concentrations in apparently healthy subjects (36,37). Thus, the relation between folate and the plasma tHcy concentration is likely to be causal. Assuming causality 3 important conclusions can be drawn from the dose-response relation between folate intake and the plasma tHcy concentration. First, it describes a curve with a slope that is somewhat steeper at its beginning but that gradually becomes flatter at higher folate intakes, similar to what was shown in other observational studies (18,20). This finding implies that an increase in folate intake [e.g. achieved by consuming an additional bowl of salad and a glass of orange juice each day (providing >50 µg folate)], has the largest effect on the plasma tHcy concentration when folate intakes are low (<200 µg/d). Second, plasma tHcy concentrations decrease at folate intakes >200 µg/d, which is the recommended dietary intake level in the Netherlands (38) and many other countries (39); thus, this intake is not sufficient to maintain an optimal low plasma tHcy concentration, as also suggested by other researchers (40-42). Finally, the curve did not

reach its nadir within this range of folate intakes; thus, the recommended intake should be >300 µg/d.

In men, we observed a stronger inverse relation between folate intake and plasma tHcy in smokers. Although it might appear that the interaction is drawn by the high mean plasma tHcy concentration at the lowest folate intake quintile in smokers, the interaction is also highly significant (P=0.002) when folate intake is analyzed on a continuos scale. In women we observed a comparable pattern in plasma tHcy concentration after stratification for smoking, but there was no statistical interaction. The interaction was not explained by a lower plasma folate concentration in smokers (32,33,43) because in our study population the plasma folate concentration in smokers was similar to that of non-smokers after correction for folate intake. The much higher plasma tHcy concentration in smokers with a similar low folate intake to that of non-smokers possibly points to the induction of a local folate deficiency in cells exposed to cigarette smoke (33). A high folate intake in smokers might secure enough folate for the remethylation of homocysteine within cells, preventing the export of excess in homocysteine to plasma.

In men the relation between folate intake and the plasma tHcy concentration was also modified by alcohol consumption. This relation was strongest in non-drinkers and absent in drinkers of >2 drinks/d. We observed no interaction in women, likely because of the small range of alcohol intake: only 42% of the women regularly drank alcohol and only 7% drank >2 drinks/d.

A priori, we expected that, in subjects with a low folate intake, alcohol drinkers would have higher plasma tHcy concentrations than would non-drinkers because of the adverse effect of alcohol on the B-vitamin status (34,35). Quite the opposite was true (figure 3). This contrast with the cited studies (in which a high amount of alcohol was studied) may have been due to the moderate alcohol consumption in our study (10% of the male drinkers in the present study consumed >5 alcoholic drinks/d). These findings add to the evidence for a potential beneficial health effect of moderate alcohol consumption on the cardiovascular system (44).

We evaluated whether the interaction was due to the type of alcohol consumed. For men in the highest alcohol category, beer was an important contributor to total folate intake. Men in the lowest folate intake quintile were more frequently wine drinkers: they drank twice as much wine (~1 drink/d) as did men in the other quintiles. Wine contains betaine (45), which serves as a methyl donor for the less common remethylation of homocysteine by betaine-homocysteine methyltransferase (restricted to liver, kidney and adrenal gland tissues) (9). This process might, at least partly, explain why these male drinkers had a low plasma tHcy concentration despite their low folate intake. We were not able to adjust for betaine intake due to the lack of dietary data for this nutrient.

We conclude that, of the B-vitamins involved in homocysteine metabolism, folate is the most important dietary determinant of the plasma tHcy concentration. In the general Dutch population studied, the Dutch folate recommendation was not met by 31% of the men and 61% of the women. It is important to note that even if all of the subjects had met the recommendation of 200 µg/d, this amount would not lead to an optimal low plasma tHcy concentration. Our study showed that a high dietary intake of folate can make a substantial contribution to a reduction in plasma tHcy concentration in the general population, which is important because each 1-µmol/L decrease in tHcy concentration may be associated with a 10% reduction in risk of cardiovascular diseases (3). These results provide an additional scientific foundation for current public health educational programs targeted at increasing the consumption of plant foods, which are particularly rich in folate, to reduce chronic diseases.

Acknowledgements

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5

The effect of the MTHFR 677 C>T polymorphism on the relation between folate intake, plasma folate and plasma homocysteine in the general Dutch adult population

Context: The common MTHFR 677 C>T polymorphism decreases the enzyme activity and hence the formation of the circulating form of folate. It has been speculated that TT subjects have higher folate requirements.

Objective: To assess the effect of this polymorphism on the relations: folate intake → plasma folate concentration → total homocysteine concentration (tHcy).

Design, Setting and Participants: Cross-sectional analyses of the above relations in a random sample (n=2051) of a population-based Dutch cohort (20-65 years).

Results: An increasing folate intake was associated with an increasing plasma folate concentration in all genotypes (CC, CT and TT). The steepest positive relation was seen in CT subjects: at a low folate intake (155 µg/d) the plasma folate resembled that of TT subjects (CT: 5.8, TT: 5.7 nmol/L), but at a high folate intake (267 µg/d) plasma folate resembled that of CC subjects (CT: 8.4, CC: 8.7 nmol/L). In TT subjects, plasma folate remained low (7.1 nmol/L) despite a high folate intake. The relation between plasma concentrations of folate and tHcy was inverse in all genotypes and most pronounced in TT subjects: at a low plasma folate concentration (4.0 nmol/L) the tHcy concentration was much higher for TT subjects than for the other genotypes (TT: 20.9, CT: 15.4, CC: 14.3 µmol/L), while at high concentrations (13.7 nmol/L) the difference in tHcy concentration was negligible. The relation between folate intake and the tHcy level also inverse and most pronounced in TT subjects.

Conclusions: Extrapolating our results shows that TT subjects need at least 10% more dietary folate than CT and CC subjects to achieve a comparable high plasma folate level and a comparable low tHcy level.

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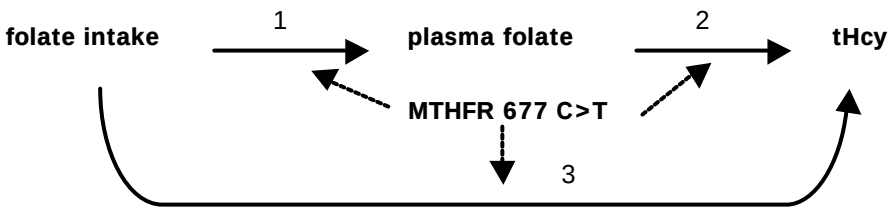
Introduction

The enzyme methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylene-tetrahydrofolate (THF) to 5-methyl-THF. 5,10-Methylene-THF is an important compound for DNA and RNA syntheses and 5-methyl-THF is the methyl donor for the remethylation of homocysteine to methionine (1,2). A common variant of MTHFR (3), characterized by a C to T missense mutation at nucleotide 677 (4), decreases the enzyme activity. The prevalence of this mutation is relatively high in the general population, e.g. the prevalence of homozygosity (TT) is 5-15% in several Caucasian populations (5), therefore it is called a polymorphism.

A lower MTHFR activity is associated with a higher plasma total homocysteine (tHcy) concentration (4,6), which is most pronounced in TT subjects with a marginal folate status (7). The polymorphism also has an effect on the distribution of folates between cells and plasma (8). Van der Put *et al* was the first to show that in TT subjects the plasma folate concentration was lower and the erythrocyte folate concentration was higher as compared with CC subjects (9). These metabolic changes are postulated to modify the risk of chronic diseases including cardiovascular diseases (10,11), cancer (12) and dementia (13-15) and neural tube defects (9,16).

Several researchers have postulated that the folate requirements of TT subjects might be higher than that of CT and CC subjects (7,17,18), nevertheless, to our knowledge no study quantified this increased need. In this study, we present data describing the effect of the 677 C>T polymorphism on the relation between folate intake and plasma folate concentration and on the subsequent relation between plasma folate and tHcy concentration. Furthermore, the effect of the polymorphism on the relation between folate intake and the tHcy concentration was investigated (figure 1).

Figure 1 Graphical presentation of the relations investigated in the present study



Explanation of symbols: 1=Relation between dietary folate intake and plasma folate concentration; 2=Relation between plasma folate concentration and plasma tHcy concentration; 3=Relation between dietary folate intake and plasma tHcy concentration: Solid arrows indicate relations, discontinuous arrows indicate effect modification.

Subjects and Methods

Subjects

We drew an age and sex-stratified random sample of 3025 subjects out of the MORGEN population examined from 1993 until 1996 (n=19,066) (19). The MORGEN study is a cross-sectional study of probability samples of men and women living in 3 Dutch towns (Amsterdam, Maastricht and Doetinchem) located in different regions in the Netherlands. The municipal population registers were used as sampling frames. The external Medical-Ethical Committee of the TNO Toxicology and Nutrition Institute, who follows the guidelines of the Helsinki Declaration, has approved the MORGEN study.

Data collection

Respondents completed two self-administered questionnaires: a general and a semi-quantitative food-frequency questionnaire. Subsequently, trained research assistants performed a physical examination at the Municipal Health Service.

From the general questionnaire we extracted information on sex, age and smoking. A semi-quantitative food-frequency questionnaire (20,21) provided information on dietary habits, from which we calculated the intake of folate and alcoholic beverages. Besides the intake of folate, we also assessed the intake of other nutrients (vitamin B2, B6, B12 and methionine) closely interrelated with folate (1,22,23). The food-frequency questionnaire did not collect information on the doses and contents of vitamin supplements. Hence, from all analyses we excluded subjects who used supplements that might have contained B-vitamins (217 men and 371 women), to reduce misclassification of B-vitamin intake.

Blood sampling and biochemical determinations

During the physical examination, non-fasting venous blood samples were obtained. The samples were collected in vacutainers containing 7.5% K3-EDTA (Safety-Monovette® tubes, Sarstedt, Tilburg, the Netherlands) and centrifuged within 1 h (10 minutes at 3000 x g). After centrifugation the plasma and red and white blood cells were separated and stored at -20°C or -80°C.

The tHcy concentration, including the protein-bound and non-protein-bound fractions of homocysteine, was measured using high performance liquid chromatography with fluorescence detection, as described by Fiskerstrand (24), with some modifications (25). The within- and between-run coefficients of variation (CV) were 3.2% and 8.6%, respectively.

Plasma folate concentration was determined by a *Lactobacillus casei* microbiological assay (26) and plasma vitamin B12 concentration was assessed by a *Lactobacillus Leichmannii* assay

(27). Both the folate and the vitamin B12 assay were adapted to a microtiter plate format and were carried out by a robotic workstation (Microlab AT plus; Hamilton Bonaduz). The within- and between- run CV's for folate were 4.3 and 10.4%, respectively, and for vitamin B12 1.1 and 1.7%, respectively.

DNA was extracted from frozen peripheral blood lymphocytes with a salting out procedure. The presence of the 677 C>T mutation was assessed by polymerase chain reaction (PCR) followed by restriction enzyme analysis with *Hin*FI (4). For 182 men and 204 women the DNA extraction or genotyping was unsuccessful. The genotype distribution was in Hardy-Weinberg equilibrium.

Statistical analysis

Due to the exclusion of B-vitamin supplement users (n=588) and subjects with a missing MTHFR genotype (n=386), all analyses are based on data of 2051 men and women.

The distributions of the plasma concentrations of tHcy, folate and vitamin B12 and the intake of all nutrients were skewed with a long tail towards higher values; therefore, natural logarithmic transformations were applied to normalize these distributions. Inverse transformations were performed to provide geometric means and their 95% confidence intervals. After logistic transformation, the intakes of the nutrients (B-vitamins and methionine) were energy-adjusted according to the method of Willett *et al* (28,29).

Mean values of B-vitamin and methionine intake and of plasma concentrations of folate, B12 and tHcy were calculated by 677 C>T genotype (CC, CT and TT) and P values for trends were calculated with univariate linear regression analyses with genotype as the independent variable.

The role of the 677 C>T polymorphism in the relations between 1) folate intake and plasma folate concentration, 2) plasma folate and tHcy concentration and 3) folate intake and tHcy concentration (figure 1) was described for men and women together, as effect modification by sex was not observed for these relations. Effect modification by the polymorphism in the above relations was evaluated in linear regression models with the inclusion of the appropriate interaction terms e.g. folate intake $\times$ genotype. A P value <0.05 for the interaction term was considered to indicate a statistically significant interaction.

Genotype-stratified relations were adjusted for age and sex, in multiple regression models we also adjusted for alcohol consumption (drinks/d) and smoking (no/yes). Furthermore, we adjusted for vitamin B12 *intake* in the relations with folate *intake* as explanatory variable, and for *plasma* B12 in the relation with *plasma* folate as explanatory variable. As we did not have plasma levels of vitamin B2, B6 and methionine we could only adjust the associations for intakes of these nutrients.

The mean adjusted plasma folate or tHcy levels (and 95% confidence intervals) for tertiles or quintiles (based on the total population, i.e. not genotype specific) of folate intake or plasma

folate levels were calculated by analyses of covariance, as implemented in SAS "PROC GLM". Differences in mean levels compared with a referent category were tested using Bonferroni's adjustment for multiple comparisons.

Findings were considered statistically significant if the two-sided P-value was <0.05. Data were analyzed with SAS statistical software (version 8.1) (SAS institute Inc., Cary, North Carolina, USA).

Results

Dietary and blood indices by MTHFR 677 C>T genotype

The study population consisted of 1094 men and 957 women with a mean age of 41 years (range 20-65 years). Dietary folate, vitamin B2, B6, B12 and methionine intakes were similar for all genotypes (table 1). The plasma folate concentration was inversely associated with the presence of a T allele. Compared with CC subjects, the average plasma folate concentration was 1.7 nmol/L lower in TT subjects and 0.8 nmol/L lower in CT subjects. The prevalence of a low plasma folate concentration (defined as <4.5 nmol/L) increased with the presence of the mutation: 8% in CC subjects, 11% in CT subjects and 23% in TT subjects. There was no association between the 677 C>T polymorphism and the plasma vitamin B12 concentration.

We observed a strong positive trend (P<0.0001) between the presence of the T allel and the tHcy level (table 1). On average, the presence of one T allele resulted in a 0.7 µmol/L higher tHcy concentration and two T alleles resulted in a 4.2 µmol/L higher tHcy level compared with subjects with no T alleles. The prevalence of hyperhomocysteinemia, (defined as tHcy >17.4 µmol/L), increased dramatically with the presence of the mutation: 8% in CC subjects, 12% in CT subjects and 38% in TT subjects.

The effect of the 677 C>T MTHFR genotype on the relations between intake of folate, plasma folate and tHcy levels

The 677 C>T polymorphism statistically significantly modified the relation between 1) folate intake and plasma folate concentration (table 2a), 2) plasma folate and tHcy concentration (table 2b), and 3) folate intake and tHcy concentration (table 2c).

Table 2a presents the age and sex-adjusted relation between folate intake and plasma folate concentration by genotype, both in tertiles and continuously. The mean plasma folate concentration increased with an increasing folate intake for all genotypes, however, more strongly in subjects with a T allele. The difference in plasma folate level between the 1st tertile (T1) and the 3rd tertile (T3) was 2.0 nmol/L for CT subjects and 1.7 nmol/L for TT subjects, compared with 1.5 nmol/L in CC subjects. On a continuous scale, in the age and sex-adjusted

models, the relation between folate intake and plasma folate level was also stronger in subjects with one or two T alleles than in subjects with no T allele (see β-coefficients in table 2a).

Table 1 Mean levels and range (1st - 99th percentile) of dietary and blood indices by MTHFR 677 C>T genotype for men and women aged 20-65 years

	all (n=2051)[a]	CC (n=983)[b]	CT (n=907)[c]	TT (n=206)[d]	P for trend
Folate intake (µg/d)	204 124-330	205 124-358	203 125-316	201 111-309	0.09
Vitamin B2 intake (mg/d)	1.53 0.88-2.93	1.53 0.86-2.92	1.52 0.88-2.93	1.53 0.90-2.82	0.8
Vitamin B6 intake (mg/d)	1.77 1.14-2.58	1.78 1.14-2.60	1.76 1.11-2.48	1.75 1.19-2.59	0.09
Vitamin B12 intake (µg/d)	4.53 1.86-12.1	4.58 1.93-12.1	4.47 1.82-11.5	4.58 2.08-12.0	0.4
Methionine intake (mg/d)	1.81 1.14-2.63	1.81 1.13-2.64	1.80 1.16-2.57	1.83 1.20-2.53	0.9
Plasma folate (nmol/L)	7.4 2.4-22.4	8.0 3.4-22.2	7.2 2.5-20.9	6.3 2.6-26.6	<0.001
Plasma vitamin B12 (pmol/L)	284 102-638	288 105-735	283 105-649	274 101-683	0.1
Plasma tHcy (µmol/L)	13.6 7.8-39.5	12.9 7.5-22.5	13.6 8.1-33.4	17.1 8.1-67.1	<0.001

Explanations of symbols: a=The minimal number of valid observations subjects was 2037; b=The minimal number of valid observations for CC subjects was 928; c=The minimal number of valid observations for CT subjects was 904; d=The minimal number of valid observations for TT subjects was 205.

The multiple adjusted (age, sex, alcohol consumption, smoking, intake of vitamin B2, B6, B12 and methionine) β-coefficients show that the relation between folate intake and plasma folate level was most strong in CT subjects. The adjusted relations are visualized in figure 2a. At a low folate intake level (1st quintile, i.e. mean intake of 155 µg/d) the plasma folate level of CT subjects was almost equal to that of TT subjects: mean plasma folate level was 5.7 nmol/L in TT and 5.8 nmol/L in CT subjects. However, at high folate intake (5th quintile, i.e. mean intake of 267 µg/d) the plasma folate level of CT subjects was almost identical to that of CC subjects: mean plasma folate level was 8.4 nmol/L in CT versus 8.7 nmol/L in CC subjects.

Table 2b shows the age and sex-adjusted relation between plasma concentrations of folate and tHcy, by genotype. The mean tHcy concentration decreased with increasing plasma folate concentration for all genotypes. This reduction was larger in CT subjects than in CC subjects (2.9 vs. 2.0 µmol/L), and largest in TT subjects (7.6 µmol/L). The age and sex-adjusted β-coefficient confirmed the strong inverse relation between the plasma folate and tHcy concentration for TT subjects on a continuous scale.

Table 2a Mean (and 95% confidence interval (CI)) plasma folate concentration per tertile (T) of folate intake, stratified by genotype

Mean folate intake per tertile	T1 166 µg/d	T2 205 µg/d	T3 250 µg/d	β[a] of folate included continuously in simple[b] model (95% CI)	β[a] of folate included continuously in multiple[c] model (95% CI)
CC, n=938[d]	7.1 (6.8;7.4)	8.2 (7.8;8.6)	8.6 (8.2;9.0)	0.47 (0.34;0.60)	0.41 (0.24;0.58)
CT, n=907[e]	6.2 (5.9;6.5)	7.3 (7.0;7.6)	8.2 (7.8;8.6)	0.68 (0.54;0.82)	0.76 (0.58;0.94)
TT, n=206[f]	5.5 (4.9;6.2)	6.4 (5.6;7.4)	7.2 (6.4;8.2)	0.77 (0.40;1.14)	0.63 (0.16;1.10)

Table 2b Mean (and 95% confidence interval (CI)) tHcy concentration per tertile (T) of plasma folate concentration, stratified by genotype

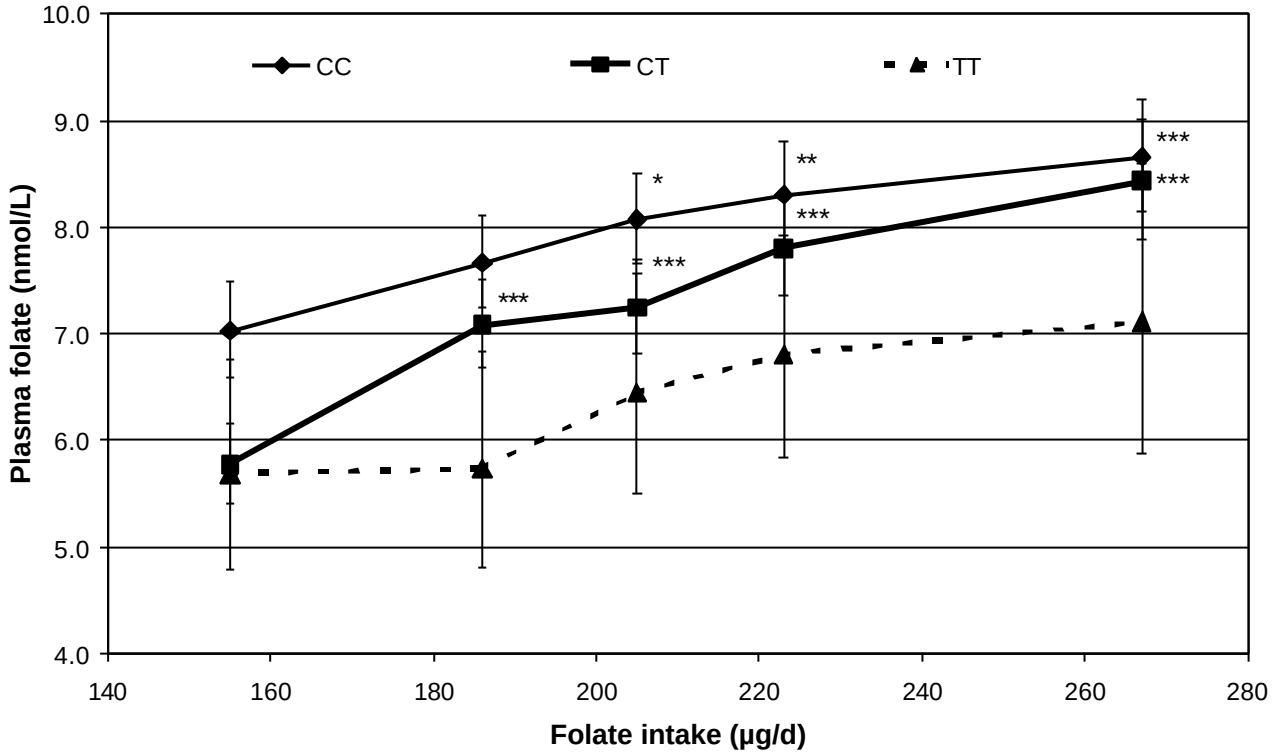
Mean plasma folate per tertile	T1 4.6 nmol/L	T2 7.5 nmol/L	T3 11.9 nmol/L	β[a] of folate included continuously in simple[b] model (95% CI)	β[a] of folate included continuously in multiple[c] model (95% CI)
CC, n=938[d]	14.1 (13.7;14.5)	12.9 (12.6;13.2)	12.1 (11.8;12.4)	-0.17 (-0.20; -0.13)	-0.15 (-0.18; -0.11)
CT, n=907[e]	15.1 (14.8;15.5)	13.2 (12.8;13.5)	12.2 (11.9;12.6)	-0.25 (-0.29; -0.22)	-0.22 (-0.25; -0.18)
TT, n=206[f]	20.6 (19.1;22.3)	14.9 (13.3;16.6)	13.0 (11.6;14.6)	-0.48 (-0.57; -0.39)	-0.38 (-0.47; -0.28)

Table 2c Mean (and 95% confidence interval (CI)) tHcy concentration per tertile (T) of folate intake, stratified by genotype

Mean folate intake per tertile	T1 166 µg/d	T2 205 µg/d	T3 250 µg/d	β[a] of folate included continuously in simple[b] model (95% CI)	β[a] of folate included continuously in multiple[c] model (95% CI)
CC, n=938[d]	13.7 (13.4;14.1)	12.6 (12.3;12.9)	12.4 (12.1;12.7)	-0.24 (-0.31; -0.16)	-0.20 (-0.30; -0.10)
CT, n=907[e]	14.6 (14.2;15.0)	13.4 (13.0;13.7)	12.8 (12.4;13.2)	-0.36 (-0.44; -0.28)	-0.30 (-0.41; -0.19)
TT, n=206[f]	19.6 (17.8;21.6)	16.3 (14.6;18.2)	15.2 (13.7;16.9)	-0.78 (-1.08; -0.49)	-0.53 (-0.90; -0.15)

Explanation of symbols: a=β's of linear regression models, they express the change in respectively log plasma folate or log tHcy associated with 1 unit change in log folate intake or log plasma folate; b=adjustments for age and sex; c=adjustments for age, sex, intake of vitamin B2, B6, and methionine, smoking (no/yes) and alcohol consumption (drinks/d). The models in table 2a and 2c were adjusted for vitamin B12 intake, and the model in table 2b for plasma vitamin B12; d,e,f=minimal number of observations d=925; e=901; f=205.

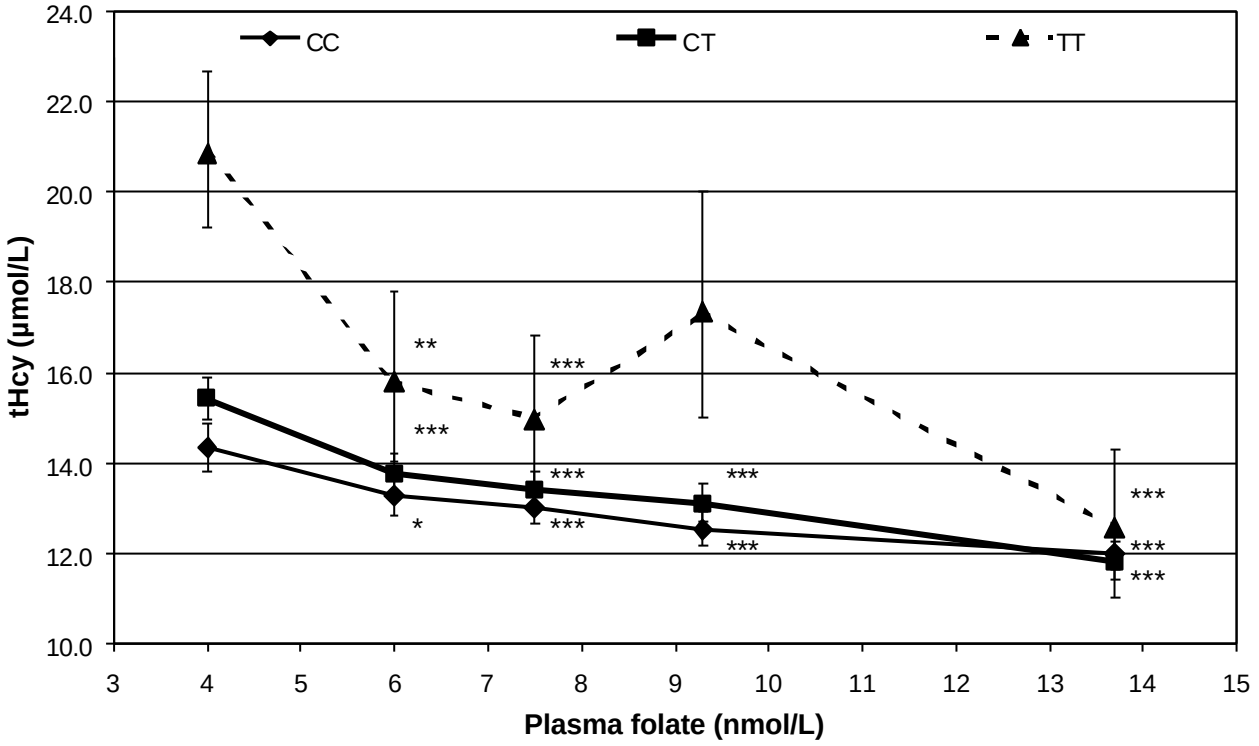
Figure 2a Mean adjusted[a] plasma folate concentration by quintiles of folate intake in Dutch men and women aged 20-65 years, stratified for genotype



Explanation of symbols: a=Means are adjusted for age, sex, smoking (no/yes) alcohol consumption (drinks/d), intake of vitamin B2, B6 and B12, methionine; Asterisks indicate significantly different plasma folate concentration compared with the first quintile of the respective genotype: * P<0.05, ** P<0.01, *** P<0.001.

After multiple adjustment the negative β-coefficient for the relation between the plasma folate (continuously) and the tHcy concentration for all genotypes did not appreciably change (table 2b). These relations are visualized in figure 2b. The effect of the MTHFR 677 C>T polymorphism was most obvious at a low plasma folate level (1st quintile, i.e. mean plasma folate of 4.0 nmol/L): TT subjects had a mean tHcy level of 20.9 µmol/L compared with 15.4 µmol/L in CT subjects and 14.3 µmol/L in CC subjects. At high plasma folate levels (5th quintile, i.e. mean plasma folate of 13.7 nmol/L) the tHcy concentration in all genotypes were similar (CC=12.0 µmol/L, CT=11.8 µmol/L, TT=12.6 µmol/L).

Figure 2b Mean adjusted[a] tHcy concentration by quintiles of plasma folate concentration in Dutch men and women aged 20-65 years, stratified for genotype

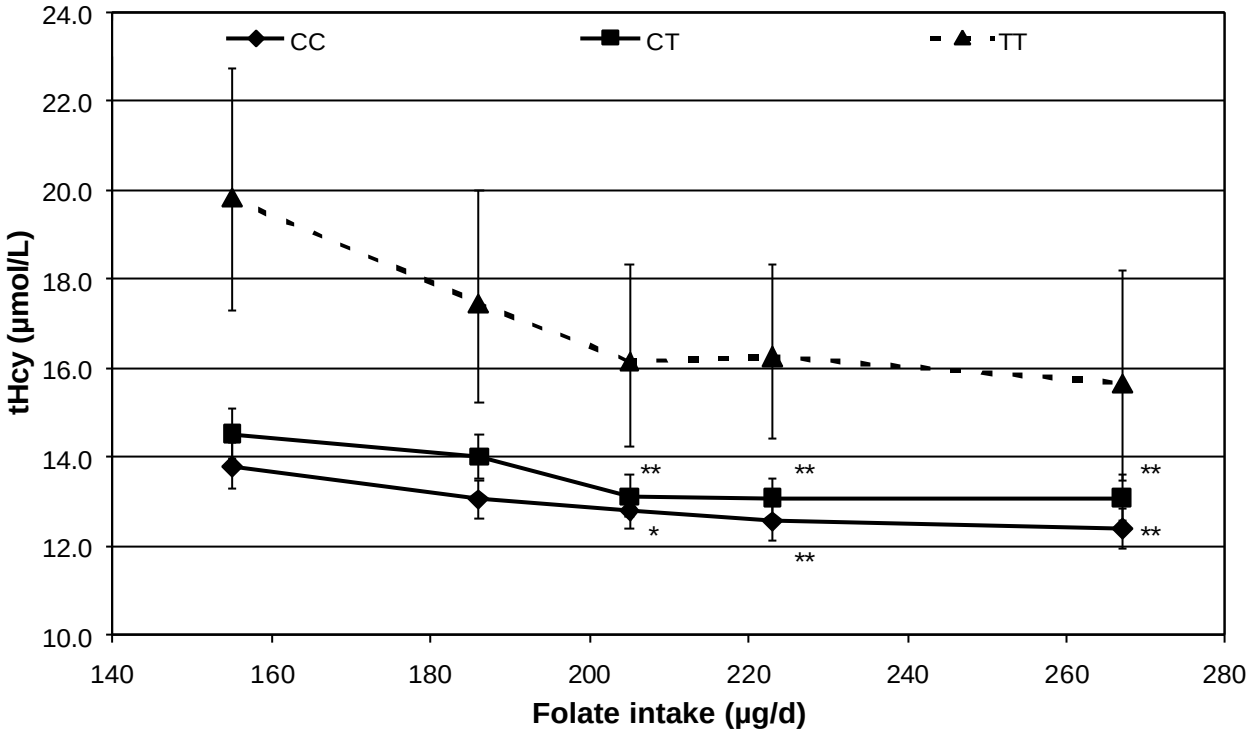


Explanation of symbols: a=Means are adjusted for age, sex, smoking (no/yes) alcohol consumption (drinks/d), intake of vitamin B2, B6 and methionine, and plasma vitamin B12; Asterisks indicate significantly different plasma folate concentration compared with the first quintile of the respective genotype: * P<0.05, ** P<0.01, *** P<0.001.

Finally, table 2c shows the age and sex-adjusted relation between folate intake and the tHcy concentration, by genotype. In general, these relations were comparable to those between the plasma folate and the tHcy concentration described above. The age and sex-adjusted β-coefficient of the relation between the concentration of plasma folate and of tHcy on a continuous scale, was strongest for TT subjects. The same is true for the multiple adjusted relation.

The β-coefficients for the relation between folate intake and the tHcy concentration were larger than those for the relation between plasma folate and tHcy concentration. This is due to the fact that extreme low or high values are more likely to occur in intake than in plasma levels. The β-coefficients of continuous relations are sensitive to these extremes. When summarizing the data in tertiles we saw that the effect of extremes disappears: the difference in tHcy concentration over the tertiles is higher for plasma folate level than for folate intake.

Figure 2c Mean adjusted[a] tHcy concentration by quintiles of folate intake in Dutch men and women aged 20-65 years, stratified for genotype



Explanation of symbols: a=Means are adjusted for age, sex, smoking (no/yes) alcohol consumption (drinks/d), intake of vitamin B2, B6, B12 and methionine; Asterisks indicate significantly different plasma folate concentration compared with the first quintile of the respective genotype: * P<0.05, ** P<0.01, *** P<0.001.

Figure 2c visualizes the multiple adjusted relation between folate intake and the tHcy level. At a low folate intake (1st quintile, i.e. mean intake of 155 µg/d) the tHcy concentration of TT subjects is statistically significantly higher than of CT and TT subjects. At a high folate intake (5th quintile, i.e. mean intake of 267 µg/d) the tHcy concentration of TT subjects was similar to that of CT subjects, but statistically significantly higher than the concentration of CC subjects (TT=15.7 µmol/L, CT=13.1 µmol/L, CC=12.4 µmol/L).

Discussion

This is the first population-based study that extensively studied the effect of the MTHFR 677 C>T polymorphism on the relation between folate intake, plasma folate and tHcy concentration. TT subjects had a lower plasma folate concentration despite the fact that their folate intake was equal to that of CT and CC subjects. CT subjects experienced an unfavorable effect of their T allele only when they had a low folate intake. The effect of the T allele in the relation between

plasma folate and tHcy level was most pronounced in TT subjects with a low plasma folate concentration. The same is true for the relation between folate intake and the tHcy level.

In the present study, 10% of the subjects were identified homozygous for the 677 C>T mutation; heterozygotes accounted for 44% of the subjects. These values are similar to those reported in other Caucasian populations (5). The fact that we had a complete data-set for a sub-sample of the original random sample (n=3025) did not substantially affect the representativity of our study sample: the distribution of age, lifestyle factors, biological cardiovascular disease risk factors (i.e. blood pressure, total and HDL cholesterol level) was equal for subjects who were (n=2051) and were not (n=974) included.

The intake of folate and other relevant nutrients was assessed with a food-frequency questionnaire validated for the intake of food groups predominantly contributing to the intake of the studied nutrients (i.e. vegetables, bread, milk and milk products, meat and potatoes) (21). Furthermore, folate intake correlated with plasma folate level in the same magnitude (Spearman correlation 0.27) as in other Dutch studies (30,31). Plasma folate levels were somewhat lower compared with levels measured in other Dutch populations (9,30,32,33). This difference may be attributable to different methods used to measure the folate concentration (34,35); in addition, we excluded all B-vitamin supplement users (see subjects and methods) and they had higher plasma folate concentrations (data not shown).

The product of MTHFR, 5-methyl-THF, is the predominant circulating form of folate, whereas the enzyme substrate 5,10-methylene-THF is found mainly intracellularly (8,36). As the 677 C>T mutation results in a reduced specific enzymatic activity of MTHFR (~34% residual activity in TT, ~71% residual activity in CT relative to CC) (37), the expected result of the presence of a T allele is a lower plasma folate concentration. After Van der Put *et al* (9) many investigators have reported a lower plasma folate concentration in TT subjects compared with CC subjects (17,38-44). We were able to investigate whether differences in folate intake could explain this observation. This was not the case: for each folate intake quintile (where folate intake was equal for all genotypes) TT subjects always had lower plasma folate concentrations compared with CT and CC subjects (figure 2a). A comparable finding was briefly mentioned in another study, but the results were not shown (17).

Extrapolating figure 2a, indicates that TT subjects would reach the average mean plasma folate level of CC subjects in the highest quintile (i.e. 8.7 nmol/L) at an average folate intake of about 375 µg/d (i.e. at an intake level 40% higher than the current mean of the 5th quintile in TT individuals). Note, however, that despite the substantial differences in mean plasma folate concentration between TT and CC subjects, these differences did not always reach statistical significance due to the relatively small number of TT subjects. Statistical power was not a problem in the larger group of CT subjects. We observed that carrying one T allele only shows a disadvantage, i.e. a low plasma folate concentration, in subjects with a folate intake in the

lowest quintile. Yet, a folate intake at or above the mean level of the 2nd quintile (i.e. >186 µg/d) resulted in similar plasma folate concentrations in CT subjects as for CC subjects. Our observation that the CT genotype can lead to low plasma folate levels when folate intake is low, may add another explanation (11) for the inconsistent results that are found when the risk of cardiovascular diseases is quantified in TT subjects versus CT and CC subjects together.

The effect of the MTHFR 677 C>T polymorphism on the relation between plasma folate and tHcy concentration (figure 2b), was most pronounced in TT subjects: in the lowest plasma folate quintile (mean plasma folate concentration: 4.0 nmol/L) TT subjects had a very high tHcy concentration compared to CT and CC subjects. On the other hand, in the highest plasma folate quintile (mean plasma folate concentration: 13.7 nmol/L) the tHcy concentration of TT subjects was equally low as that of CT and CC subjects. A statistically significant difference in mean tHcy concentration between the genotypes was no longer present at an average plasma folate concentration of 7.5 nmol/L (3rd plasma folate quintile). From figure 2a we can deduce that TT subjects would reach this average plasma folate concentration at a daily folate intake of ~300 µg/d, a level 10% higher than the current mean of the 5th quintile in TT individuals. Note that this is probably an optimistically low estimation as only in the highest plasma folate quintile the tHcy concentrations of the genotypes were similar (figure 2b). From figure 2c it can be deduced that when TT subjects would have a mean intake level of 300 µg/d, this would lead to a lower tHcy concentration, approaching the level of CC subjects. Yet, to achieve a similarly low tHcy concentration, a folate intake of more than 300 µg/d is probably necessary.

The effect of the polymorphism on the relation between the plasma folate and tHcy concentration agrees with other studies (7,11,45). So far, one other study has reported the modifying effect of the polymorphism on the relation between folate intake and the tHcy level (17). Similar as in the present study, they found that at a low folate intake TT subjects had much higher tHcy levels compared with CT and CC subjects. Alternatively, at high folate intakes the tHcy level of TT subjects was more comparable to that of CT and CC subjects. These results in general population samples agree with the results found in folic acid intervention studies. These trials showed a steeper decrease in tHcy concentration as a response to an increased folate intake in TT subjects compared with CT and CC subjects (39,46).

The mechanism behind the observation that a high folate intake protects against high tHcy concentrations and low plasma folate levels in subjects with a T allele is nicely illustrated by recent findings of Guenther *et al* (23). In a system with bacteria they showed that a mutation homologous to the human MTHFR 677 C>T mutation was associated with an enhanced dissociation of flavin adenine dinucleotide (FAD, i.e. cofactor form of vitamin B2). An optimal folate supply prevented the loss of FAD and suppressed the inactivation of the enzyme (23).

In conclusion, this study describes an important gene-environment interaction in the general population. Our findings indicate that subjects with the 677 C>T mutation, especially those with

the mutation in homozygous form, have a higher dietary folate requirement to obtain a plasma folate concentration comparably high as that of CT and CC subjects. Once they have reached a high plasma folate concentration, their tHcy concentration is not different from that of CT or CC subjects. Currently, the recommended dietary intake of folate for adults is 200–300 µg/d in The Netherlands (47) and other European countries (48). This recommendation is estimated to be sufficient to cover the needs of the majority (97.5%) of the healthy population. This study demonstrated that 10% of the population, with the TT genotype of the MTHFR 677 C>T polymorphism, requires more folate than 300 µg/d to optimize their plasma folate and tHcy concentration. In light of our results, a reconsideration of the Dutch recommendations according to the recent new dietary reference intakes in the United States (i.e. 400 µg of folate/d) (49) may be appropriate.

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6

Lifestyle factors and plasma homocysteine in the general Dutch adult population

The authors cross-sectionally investigated the extent to which coffee, tea, and alcohol consumption, physical activity, and smoking were associated with the non-fasting total plasma homocysteine (tHcy) concentrations in a random sample of 3025 Dutch adults aged 20-65 years from a population-based cohort examined in 1993-1996 (n=19,066).

The lifestyle factors most strongly associated with the tHcy concentration were smoking (positive), alcohol drinking (negative), and coffee consumption (positive). The smoking effect was most prominent in women, and the alcohol effect was most pronounced in men. Data indicated that independently of other lifestyle factors, age, and intake of folate and B-vitamin supplements, a change in lifestyle could result in a 0.1 to 1.7 µmol/L change in the tHcy concentration. The authors conclude that lifestyle changes could result in a public health relevant change in the tHcy concentration.

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Introduction

From a public health viewpoint it is important to identify modifiable factors that influence the plasma total homocysteine (tHcy) level, because elevated concentrations are associated with an increased risk of cardiovascular diseases (1,2). Folate intake is identified as the most important dietary determinant of the tHcy concentration (3,4). Besides diet, other lifestyle factors such as smoking (5), physical activity (6), and consumption of alcohol (7-9), coffee (5,10-13), and tea (10) may have an effect on tHcy levels in the general population. Inconclusive results for alcohol and coffee consumption and a lack of representative populations with a broad age range creates the need for further investigation. In addition, only two studies have corrected for the confounding effect of folate intake (7,11). Using a representative sample of the general Dutch population aged 20-65 years, we studied the association of these lifestyle factors with tHcy concentrations, taking folate intake into account.

Materials and methods

The subjects and methods of this study have been described extensively elsewhere (14). Briefly, we drew an age- and sex- stratified random sample (n=3025) of subjects in a population-based cohort that was examined during 1993-1996 (n=19,066) (15).

Data were collected with self-administered questionnaires and a physical examination. On the basis of responses to questions about smoking, we categorized subjects as either non-smokers or light (<10 cigarettes/d), moderate (≥10 - <20 cigarettes/d) or heavy (≥20 cigarettes/d) smokers. Physical activity was reported as the average amount of time (minutes/week) spent in various activities during leisure time, work time, household activity, and commuting (e.g., cycling to work) over the previous year. For the statistical analyses, we defined 4 levels of weekly activity: “sedentary”: <0.5 h; “moderately active”: ≥0.5 - <3.5 h; “active”: ≥3.5 h of which ≤2 h heavy activity; and “very active”: ≥3.5 h of which >2 h heavy activity (16). Information from subjects participating in 1993 (n=408) was not included, since the questions on activity in 1993 were different from those posed in 1994-1996.

A semi quantitative food-frequency questionnaire (17,18) provided information on the consumption of alcohol, coffee, and tea, which was recoded into units per day. Energy-adjusted folate intake was estimated as described elsewhere (4).

The tHcy concentrations from non-fasting venous blood samples were determined as described by Fiskerstrand *et al* (19), with some modifications (20).

We used the SAS software (version 6.12) for all statistical analyses (SAS Institute, Inc., Cary, North Carolina). Comparisons between men and women were conducted using Wilcoxon’s 2-sample tests for continuous variables and chi-squared tests for proportions. The tHcy

distribution was normalized by logarithmic transformation. Thus, geometric means are presented unless stated otherwise. Each lifestyle-tHcy association was evaluated by univariate and multivariate linear regression. Differences in adjusted mean tHcy concentrations as compared with a reference category were tested using analyses of covariance. In the multivariate models, we adjusted for age (years), folate intake (µg/d), intake of B-vitamin supplements (no/yes), and the other studied lifestyle factors. When considered as confounders, the lifestyle factors were included continuously, except for smoking, which was included as 3 indicator variables (light, moderate, and heavy smoking) with non-smokers used as the reference group.

Results

Age and the prevalences of inactivity and smoking were equal for men and women (table 1). Women had a lower tHcy concentration, folate intake, alcohol and coffee consumption than men but a higher tea consumption than men.

Table 1 Selected characteristics of Dutch men and women aged 20-65 years examined in 1993-1996

Characteristics	Men (n=1493)[a]		Women (n=1532)[a]	
	Mean	SD	Mean	SD
Age (years)	40.5	12.1	41.0	12.5
tHcy (µmol/L)[b]	14.6	6.1	13.1*	4.6
Folate intake (µg/d) [b]	240	75	194*	53
Coffee (cups/d)	4.9	3.2	4.1*	2.8
Tea (cups/d)	1.5	1.9	2.1*	2.4
Alcohol (glasses/d)	1.9	2.3	0.7*	1.1
Less than 30 min of moderate or heavy physical activity/week (%)	21.7		20.9	
Current smokers (%)	35.4		37.3	

Explanation of symbols: a=The minimal number of valid observations for men was 1488 and for women 1529, except for physical activity: where the percentages were based on 1297 men and 1319 women; b=Arithmetic value; SD=standard deviation; Asterisks indicate a statistically significant difference from males P<0.001.

Univariate linear regression (table 2), in which the beta coefficients express a proportional change in tHcy level because of the logarithmic transformation, showed that in both men and women coffee consumption was positively associated, tea consumption was negatively associated, and physical activity was not associated with the tHcy concentration. Alcohol consumption showed a negative association with the tHcy concentration in men only, and smoking showed a positive association, in women only.

Table 2 Proportional change and 95% confidence interval (CI) in the tHcy concentration associated with a one-unit change in various lifestyle factors among Dutch men and women aged 20-65 years examined in 1993-1996

	Men				Women			
	Univariate n=1493[a]		Adjusted[b] n=1287[a]		Univariate n=1532[a]		Adjusted[b] n=1314[a]	
	%	95% CI	%	95% CI	%	95% CI	%	95% CI
Coffee (cups/d)	0.77	0.32, 1.22	0.48	-0.02, 0.99	1.67	1.20, 2.14	1.03	0.46, 1.60
Tea (cups/d)	-1.03	-1.77, -0.29	-0.43	-1.25, 0.38	-1.00	-1.56, -0.44	-0.18	-0.80, 0.44
Alcohol (glass/d)	-1.15	-1.77, -0.54	-1.06	-1.71, -0.41	-0.16	-1.40, 1.08	-0.47	-1.77, 0.82
Low folate intake			-1.52	-2.73, -0.31				
High folate intake			-0.79	-1.52, -0.06				
Physical activity[c]	0.39	-1.08, 1.86	1.05	-0.44, 2.54	0.34	-1.16, 1.84	1.71	0.25, 3.16
Non-smoking vs. smoking	1.79	-1.19, 4.77	0.84	-2.38, 4.07	6.21	3.45, 8.97	3.56	0.52, 6.59

Explanation of symbols: a=The minimal number of valid observations for men was 1488 and for women 1529, except for physical activity: the data were based on 1297 men and 1319 women. Multivariate results were based on a minimum of 1287 men and 1314 women; b=Adjustments were made for age, folate and B-vitamin supplement intake and the other lifestyle factors mentioned in this table; in the analyses where smoking was considered as confounder we adjusted with indicator variables with non-smokers as reference; c=Refer to materials for definition of categories.

The multivariate results showed essentially the same associations, except that the inverse relation with tea disappeared and activity level became positively associated in women. In table 2, results for alcohol consumption in men are also presented stratified for folate intake (below and above the median intake level of 204 µg/d), because of the interaction between alcohol and folate intake (4). Smoking also showed an interaction with folate intake in men (4), demonstrating a significant positive association among men with folate intakes below the first quintile (i.e., <176 µg/d). We did not stratify the results for folate intake, since this would only have affected the power of the analyses, whereas the direction of the association between smoking and tHcy remained positive.

The adjusted results for coffee, alcohol, and smoking are displayed in figures 1-3 respectively. Drinking >6 cups of coffee/d was associated with a 1.4 µmol/L higher tHcy concentration in men and a 1.1 µmol/L higher concentration in women, as compared with coffee abstainers (figure 1). Drinking >2 glasses of alcoholic beverages/d was associated with a 1.7 µmol/L lower tHcy level in men with a low folate intake (≤204 µg/d) and a 0.8 µmol/L lower level in men with a high folate intake (>204 µg/d), as compared with alcohol abstainers (figure 2). Women who smoked heavily had a 0.8 µmol/L higher tHcy concentration than women who did not smoke (figure 3).

Figure 1-3 Mean adjusted plasma total homocysteine (tHcy) concentration according to lifestyle among Dutch men (◆) and women (■) aged 20-65 years examined in 1993-1996

Figure 1 Coffee consumption

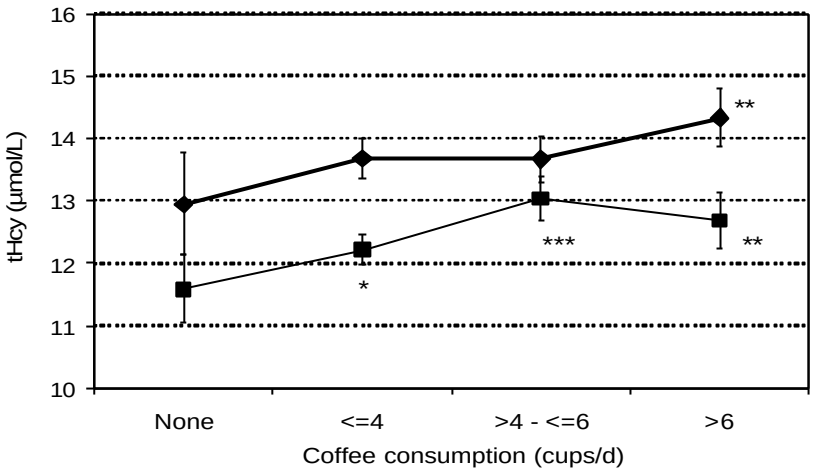


Figure 2 Alcohol consumption

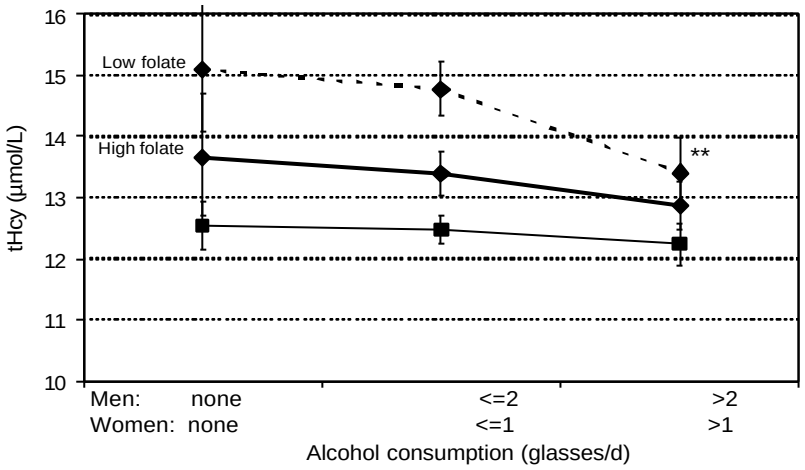
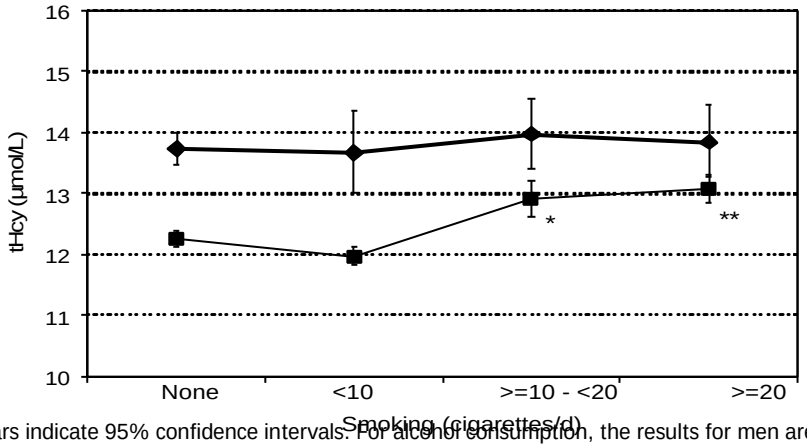


Figure 3 Smoking



Explanations of symbols: The error bars indicate 95% confidence intervals. For alcohol consumption, the results for men are stratified to folate intake. The means are adjusted for age, tea consumption, physical activity, folate and B-vitamin supplement intake and the other lifestyle factors in this figure; in the analyses where smoking was considered as confounder we adjusted with indicator variables with non-smokers as reference. Asterisks indicate statistically significant different tHcy concentration in comparison with the first category: * P<0.05, ** P<0.01, *** P<0.001.

Discussion

In this study, smoking and coffee and alcohol consumption were associated with tHcy concentrations, independently of other lifestyle factors, age, use of B-vitamin supplements, and folate intake, the latter being the major dietary determinant of the tHcy concentration. The effect of smoking was most pronounced in women, and the alcohol effect was most pronounced in men.

The positive dose-response relation that we found between coffee consumption and the tHcy concentration is consistent with most (10-12) but not all (13) observational studies. Reservations about the effect of coffee on the tHcy level have been refuted by recent intervention studies (21,22). The randomized crossover designs of the latter studies increased the likelihood of there being a causal relation between coffee consumption and the tHcy concentration.

The intervention studies showed similar effects for filtered coffee (1.5 µmol/L) (21) and unfiltered coffee (1.2 µmol/L) (22), indicating that the tHcy-raising factor is not removed by the use of paper filters. Caffeine is a proposed factor (10,22) as it might obstruct the conversion from homocysteine to cysteine by acting as a vitamin B6 antagonist (22). Therefore, the null finding for tea in the present study was not unexpected, since the amount of caffeine in tea is small (23). Intervention studies with assessment of both decaffeinated coffee and regular coffee would provide more insight on this topic.

High levels of alcohol consumption increase the tHcy concentration (24,25); however, we studied moderate alcohol consumption (16% of our male drinkers consumed ≥4 glasses/d). An inverse relation between moderate alcohol consumption and the tHcy concentration has been observed previously in men from Caerphilly (7) and in Norwegian men and women (8). However, as in the present study, no statistically significant association was found among US women in another study (9); this could be due to the small range of alcohol intakes in women.

The fact that we observed no association in women could indicate a dose effect of ethanol. On the other hand, the type of alcoholic beverage consumed could be important (25). Beer was the most frequently consumed alcoholic beverage among men. Beer contains vitamins that serve as cofactors in homocysteine metabolism, i.e. folate, vitamin B2 and B6. Although residual confounding cannot be excluded, beer consumption was significantly inversely associated with the tHcy concentration independently of these vitamins. This might point to ethanol or another substance in beer (other than B-vitamins) being the tHcy-lowering factor. Betaine, which is present in wine (26), could also have contributed to the overall inverse relation between alcohol and the tHcy level. Betaine is used in a metabolic route independent of folate to methylate homocysteine to methionine (27). Because of the lack of dietary data on betaine we were unable to adjust for it.

In the multivariate analyses, we observed a weak positive relation between physical activity and the tHcy concentration in women. This contrasts with the protective effect observed in the Hordaland Homocysteine Study (6). Since activity is generally associated with a healthier lifestyle (28), our finding is the opposite of what would be expected. Intervention studies might be able to elucidate the effect of activity on the tHcy concentration.

In accordance with other population-based studies (6,9) smoking was positively associated with the tHcy level, the effect being clearest in women. The mechanism behind the increase is unidentified. Smokers often have a lower plasma folate status than non-smokers (29-31); however, in the present study, this was explained by a lower folate intake. Thus, smoking does not seem to reduce the availability of folate for the remethylation of homocysteine to methionine. This suggests that smoking either induces local effects in cells exposed to cigarette smoke (30), or changes in the plasma thiol redox status (homocysteine is an aminothiol) (32) by a higher formation of reactive oxygen species (33,34), or inhibits the action of enzymes such as methionine synthase (35).

Subjects participating in surveys like ours might be more health conscious than non-participants. All subjects invited to participate in the MORGEN study (15) received a response card; approximately 70 percent returned the response card, while approximately 50 percent completed the full assessment. A non-response study indicated that non-responders were more likely to be men, smokers, non-drinkers of alcohol, and physically inactive. However, a comparison of the distributions of date on sex, lifestyle, and demographic factors in our random sample with nationwide data (36) showed similar percentages (14). Therefore, our results may be applicable to a wider population of Dutch men and women.

In summary, smoking, and alcohol and coffee consumption were major lifestyle correlates of the tHcy concentration. It is important to increase the public's awareness that feasible changes in lifestyle may favorably alter the tHcy concentration, which in turn may reduce the risk of cardiovascular diseases.

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Type of alcoholic beverage and plasma homocysteine in the general Dutch adult population

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Letter to the editor

Several recent publications (1,2) have reported on a potential beneficial health effect of moderate alcohol consumption on the cardiovascular system. We would like to complement these reports with data on the relation between alcohol consumption and the plasma total homocysteine (tHcy) concentration. A high tHcy concentration is associated with an increased risk of cardiovascular diseases, therefore, it is important to know how lifestyle factors might influence the tHcy concentration.

Observational studies indicate that alcohol consumption might be related to the tHcy concentration in a J-shaped fashion (3): alcoholics have a very high tHcy concentration (4) and moderate alcohol consumers (≤4 glasses/d) have a lower tHcy concentration compared with non-drinkers (3,5). In our study population, a random sample (n=3025) of a population-based cohort of Dutch men and women (20-65 years) (6), we cross-sectionally observed lower tHcy concentrations at higher levels of alcohol consumption (assessed with a food-frequency questionnaire). This trend was statistically significant in men ($P<0.001$); non-drinkers (n=132) had a (geometric) mean tHcy concentration of 14.2 µmol/L, compared with 13.9 µmol/L in drinkers of ≤2 glasses/d (n=838), 12.5 µmol/L in drinkers of >2-<4 glasses/d (n=306) and 13.1 µmol/L in drinkers of ≥4 glasses/d (n=214). An overall statistically significant inverse trend ($P<0.05$) remained after correction for age, smoking, physical activity, coffee and tea consumption, dietary folate intake and use of B-vitamin supplements.

An intriguing question is whether the inverse relation can be ascribed to ethanol intake or that the type of alcoholic beverages consumed is important, as the recent intervention trial of Van der Gaag *et al* (7) suggests. They showed, in a 3 week randomized cross-over trial, that despite the equally administered amount of ethanol (4 glasses/d=40 g/d) beer does not affect the tHcy concentration, whereas wine and spirits induce an increase. Motivated by these results (7) we studied whether different types of alcoholic beverages were differently related to the tHcy concentration in males (table 1). We found that higher beer consumption was inversely associated to the tHcy concentration, whereas wine (red and white) and spirits showed no relation with the tHcy concentration. Thus, like Van der Gaag *et al* (7), we showed that beer drinking does not have an adverse effect on the tHcy concentration. In fact, we observed a favorable effect, which may seem inconsistent. However, as the relation between alcohol consumption and the tHcy concentration might be J-shaped (3) and our study contained few heavy drinkers (16% of the male drinker drank >4 glasses/d) we were probably measuring an effect in the descending part of the J-curve. The intervention trial provided relatively high alcohol doses (7) and might have measured an effect at or beyond the nadir of the curve.

Table 1 Mean[a] plasma homocysteine (tHcy) concentration in male alcohol drinkers by tertiles of beer, wine and spirits

Tertile	Beer (n=1179)				Wine (n=1179)				Spirits (n=1179)			
	N	Intake (g/d)	Glasses/d[b]	tHcy[c]	N	Intake (g/d)	Glasses/d[b]	tHcy[d]	N	Intake (g/d)	Glasses/d[b]	tHcy[e]
1	423	20	0.1	14.3	398	0	0	13.6	579	0	0	13.8
2	387	177	0.9	13.8	385	6	0.06	13.8	208	1	0.02	13.7
3	369	747	3.7	13.0	396	87	0.9	13.7	392	33	0.7	13.6
Beta (95% CI)[f]:	-0.007 (-0.011,-0.003)%				-0.003 (-0.022, 0.016)%				0.026 (-0.021,0.074)%			

Explanation of symbols: a=Geometric means: anti-log of the logarithmically transformed tHcy values, to normalize the distribution; b=Each glass contains about 10 g of ethanol; c,d,e=tHcy adjusted for age, coffee and tea consumption, smoking, physical activity, dietary folate intake and B-vitamin supplement, other alcoholic drinks, alcohol free beer and: c=dietary vitamin B6 and B2 intake, wine and spirits, d=beer and spirits, e=beer and wine. Due to missing values in covariables, the multivariate analyses (analyses of covariance) were based on data of 1174 male alcohol drinkers; f=The beta's of the regression analysis express a proportional change in tHcy, due to the logarithmic transformation (95% CI=95% confidence interval). For example, an increase of 200 grams of beer (1 normal glass) is associated with a decrease in tHcy of 1.4%.

The beneficial effect of beer drinking on the tHcy concentration could be due to its folate, riboflavin and vitamin B6 content, all important for the enzymatic homocysteine conversion. Nevertheless, the inverse relation with beer was independent of these nutrients (table 1), which might indicate a dose effect of ethanol. This is further suggested by the absence of a significant association with alcohol consumption in female drinkers, who on average drink less then men, and the fact that the amount of ethanol consumed in the third tertile (T3) of beer consumption exceeds that of T3 in the wine and spirit drinkers by far. Intervention studies with moderate amounts of ethanol (<40 grams/d), or observational studies in populations where beer is not the predominant alcoholic drink, may elucidate whether our result is due to residual confounding of B-vitamins in beer, or that ethanol is responsible for the beneficial effect of moderate ethanol consumption on the tHcy concentration. Acknowledgements

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Biological cardiovascular risk factors and plasma homocysteine in the general Dutch adult population

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Letter to the editor

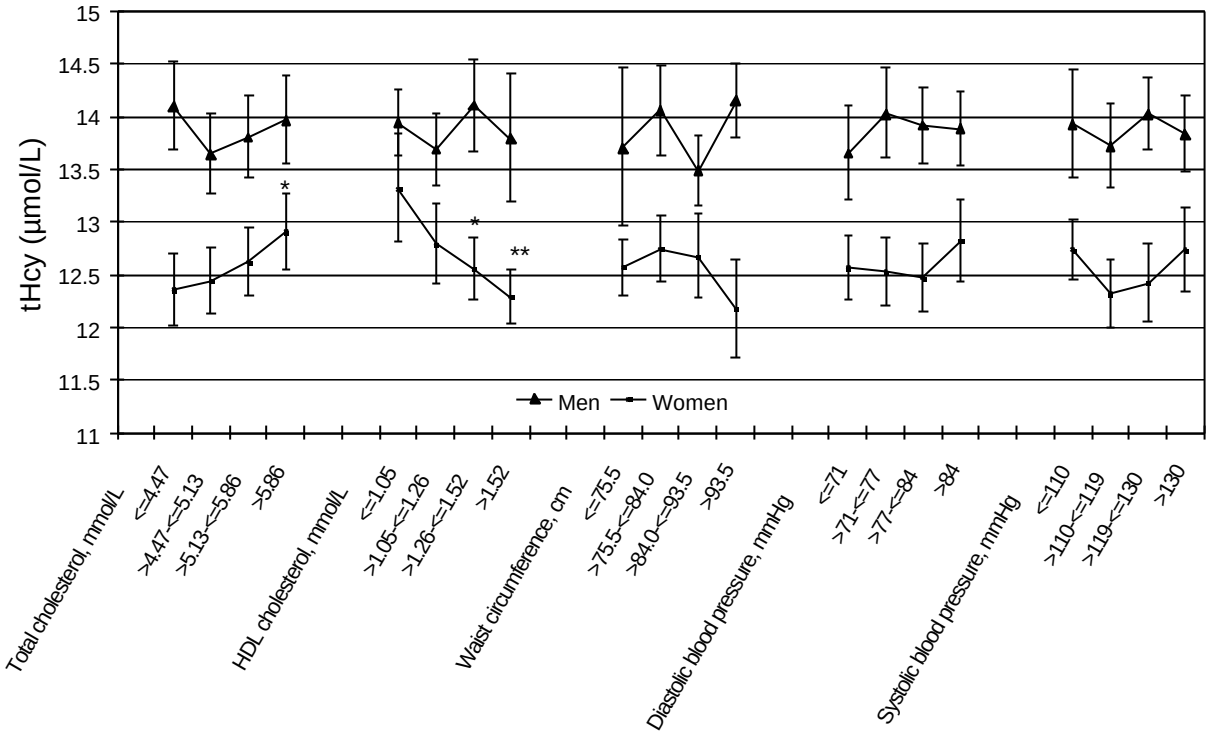
A raised plasma total homocysteine (tHcy) concentration is considered to be a risk factor for cardiovascular diseases (CVD) (1). However, evidence provided by prospective studies is weaker than that offered by case-control and cross-sectional studies (2). In addition, a genetic defect resulting in elevated tHcy concentrations is not consistently associated with the risk of CVD (3). This has raised the question whether the observed association between the tHcy concentration and CVD can be attributed to confounding by biological CVD risk factors (2,3). In this study we describe the association between cholesterol (total and HDL), blood pressure (diastolic and systolic) and waist circumference and the tHcy concentration.

We performed a sub-study on 1493 men and 1533 women within a population-based cross-sectional study of 19,066 subjects aged 20-65 years randomly selected from three Dutch towns. Data were collected with self-administered questionnaires and a physical examination, which included the drawing of blood. Standard validated techniques were used for biochemical analyses and physical measurements.

As the distribution of the non-fasting tHcy concentration was positively skewed, we log-transformed the values and present geometric means. Mean tHcy concentration was higher in men (13.9 µmol/L) than in women (12.6 µmol/L). Linear regression analyses only showed a weak inverse relation between HDL cholesterol and the tHcy concentration in men (P=0.09). In women, all risk factors were unfavorably associated with tHcy concentrations (P for all beta's <0.2). Multivariate linear regression analyses only revealed associations in women: each mmol/L increase in total cholesterol was associated with a 1.9% (P=0.008) increase in tHcy concentration and each mmol/L decrease in HDL cholesterol was associated with a 3.9% (P=0.04) higher tHcy concentration. Adjustments were made for age (years), coffee (cups/d) and alcohol (glasses/d) consumption, smoking (no/yes), folate intake (µg/d), B-vitamin supplement use (no/yes) and the other studied biological risk factors (for blood pressure we used systolic blood pressure). The multivariate relations are shown in figure 1.

Logistic regression was used to calculate multivariately adjusted odds ratio's (OR) for hyperhomocysteinemia (defined as tHcy >17.4 µmol/L). The OR for subjects with hypertension (diastolic ≥95 mmHg and/or systolic ≥160 mmHg) was 1.21 (95% confidence interval (CI)=0.69-2.12) in men and 1.92 (95% CI=0.96-3.84) in women, for subjects with a high total/HDL cholesterol ratio (>5) 0.83 (95% CI=0.58-1.17) in men and 1.78 (95% CI=1.10-2.89) in women, and for subjects with a high waist circumference (men: ≥94 cm, women: ≥80 cm) 1.27 (95% CI=0.89-1.81) in men and 0.85 (95% CI=0.56-1.29) in women.

Figure 1 Mean adjusted[a] tHcy concentration according to quartiles[b] of biological CVD risk factors.



Explanation of symbols: Error bars indicate the 95% confidence intervals; a=Means are estimated with analysis of covariance: "PROC GLM" in SAS (version 6.12). Adjustments were made for age (years), coffee (cups/d) and alcohol (glass/d) consumption, smoking (no/yes), folate intake (µg/d), B-vitamin supplement use and the risk factors in this figure; b=The quartiles were based on the distribution of the whole population; Asterisks indicate a tHcy concentration that is significantly different from the concentration in the first quartile: * P<0.05, ** P<0.01.

In this population-based study, biological risk factors for CVD were not related with the tHcy concentration in men. However, in women, an adverse cholesterol profile was significantly associated with a 78% increased risk of hyperhomocysteinemia, and hypertension tended to be associated with a comparable increase in risk (92%). Another population-based study has observed associations with cholesterol level and blood pressure in men as well as in women, which could be due to overall larger power of that study and the large proportion of subjects between 65-67 years (4).

It is unlikely that the associations observed in this study are the result of the insulin resistance syndrome (5), as we found no relation with waist circumference. In addition, most pronounced relations were seen in women, which might point to an effect of estrogens. Alternatively, lifestyle could also be an explanation. Briefly, the few observed associations with biological CVD risk factors are in the direction of an increased risk of CVD. Therefore, inaccurate control for biological risk factors could falsely increase the risk estimate in studies that quantify the relation between the tHcy concentration and CVD. Nevertheless, confounding of biological

CVD risk factors can probably not fully explain the frequently observed relation between tHcy concentrations and CVD.

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Plasma homocysteine, B-vitamins and the risk of coronary heart disease mortality in the general Dutch adult population

Background: Elevated plasma total homocysteine (tHcy) levels and B-vitamin status are associated with the risk of coronary heart disease (CHD). So far, results from prospective studies have been inconclusive.

Objective: To determine the relations between plasma concentrations of tHcy, folate, vitamin B6 (PLP) and B12 and CHD mortality.

Design: Prospective case-cohort study.

Setting: A population-based cohort of ~36,000 Dutch adults aged 20-59 years at baseline.

Participants: A random sample from the cohort (n=630) complemented with all subjects who died of CHD (n=102) during a mean follow-up of 10.3 years.

Measurements: Plasma tHcy, folate, PLP and vitamin B12 concentrations were determined. CHD mortality was identified through linkage with the national mortality register of Statistics Netherlands.

Results: Men with a tHcy level in the highest tertile (T3) compared with men in the lowest tertile (T1) had a relative risk (RR) of CHD of 1.14 (95% confidence interval (CI)=0.50-2.61) after adjusting for age, study center, hypertension, HDL and total cholesterol, smoking and creatinine. For women this RR was 2.04 (95% CI=0.48-8.62). In women only, high folate levels were associated with a statistically significant protection of fatal CHD (T3 vs. T1: RR: 0.22, 95% CI=0.06-0.87). Plasma PLP and vitamin B12 concentrations were not associated with CHD risk.

Conclusions: Elevated tHcy levels are not a major risk factor for CHD mortality in this population sample of relatively young Dutch subjects. Of the plasma B-vitamins only folate concentrations in women were associated with a lower risk of CHD mortality.

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Introduction

About 30 years ago McCully and Wilson (1) postulated that moderately elevated plasma total homocysteine (tHcy) concentrations were causally related to the development of coronary heart disease (CHD). Although initially numerous case-control and cross-sectional studies supported this hypothesis (2), nowadays several issues have led to a more cautious approach of elevated tHcy concentrations being a causal risk factor for CHD. The first concern is that the exact mechanism that underlies this association is not understood (3). Furthermore, a genetic defect in the gene encoding for MTHFR (a key enzyme in homocysteine metabolism), resulting in elevated tHcy concentrations is not consistently associated with the risk of CHD (4). Finally, prospective epidemiological studies, which compared with case-control and cross-sectional studies have a stronger methodological design to identify causal relationships, show less convincing evidence for the hypothesis (5). Thus, pending intervention trials in which the tHcy concentration is lowered with folic acid, and other B-vitamins (6), proof of a causal relation between the tHcy concentration and CHD is not available.

Of the prospective studies that have been performed in populations not selected on the basis of their risk of CHD, a small majority showed an independent statistically significant association between elevated tHcy levels and the development of CHD (7-15). Others found associations that were compatible with an increased risk, but lacked statistical significance (16-18) and a minority found no association (19-23). These inconsistencies could partly derive from different lengths of follow-up and from differences in the study populations.

We prospectively studied the relation between the tHcy concentration and the risk of CHD mortality after 10.3 years of follow-up, in Dutch adult (20-59 years) men and women free of baseline cardiovascular diseases. In addition, we also investigated plasma concentrations of folate, vitamin B6 (PLP) and B12 in relation to CHD mortality. These B-vitamins, which are important in homocysteine metabolism, may be inversely associated with CHD (16,24-27).

Methods

Study population

From 1987 through 1991 the Monitoring Project on Cardiovascular Disease Risk Factors collected information on more than 36,000 men and women aged 20-59 years from 3 Dutch towns (Amsterdam, Doetinchem and Maastricht). All participants were physically examined and filled out a self-administered questionnaire (28).

Baseline measurements

During the physical examination the systolic and diastolic blood pressure was measured twice on the left arm using a random zero sphygmomanometer, while the subject was seated. We used the mean of the two measurements in our analyses. Weight and height were measured to calculate the body mass index (BMI; kg/m^2), an index of relative weight.

The self-administered questionnaire provided information on demographic variables (e.g. education), history of cardiovascular diseases, history of diabetes, medication and several lifestyle and dietary habits. Educational level, measured with a standardized questionnaire, was classified into three categories: low (intermediate secondary education or less), medium (intermediate vocational or higher secondary education) and high (higher vocational or university education). A history of cardiovascular diseases was defined as a positive answer on the questions: 1) "Are you medically treated by a physician or specialist for heart problems?" 2) "Did you ever experience a myocardial infarction?" 3) "Did you undergo heart surgery?" and 4) "Did you ever experience a cerebrovascular accident?" The question: "Do you have diabetes?" was used to define a history of diabetes. Hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg or the use of anti-hypertensive medication. Smoking habits were dichotomized into non-smoking and smoking (≥ 1 cigarette/d). The amount and type of alcoholic beverage was recoded into total alcohol consumption in glasses/d. Coffee consumption was recorded in cups/d. Vitamin supplement use was requested in categories, e.g. vitamin C, multivitamins, B-vitamins complex, etc. The questionnaire did not ask for the vitamin dose or for the exact frequency with which supplements were taken. From these categories we created a variable which indicated whether a subject used supplements containing B-vitamins or not (note that folic acid was not commonly present in supplements at the time of baseline measurements). Participants scored physical activity separately for activity during work and leisure time. Of these scores we created one physical activity variable with three levels: 1) inactive, i.e. little or no exercise during leisure time and/or work time, 2) regularly active, i.e. regular exercise during leisure time (~4 h/week), no physical activity during work, and 3) regular strenuously active, i.e. regular (strenuous) exercise during leisure time, and/or frequent or continuously physically strenuous work. This categorization correlated positively with HDL cholesterol levels.

Mortality follow-up

The mortality follow-up lasted until January 1, 2000 (minimum of 8 years and maximum 13 years). Information about the vital status was obtained from the municipal population registry in the town of residence. If a person had moved with unknown destination, the date on which the person's name was removed from the municipal population register was used as a censor

date. For our population, 0.02% was lost to follow-up. Causes of death were identified through linkage with Statistics Netherlands.

Causes of death were coded according to the ninth revision of the International Classification of Diseases (ICD-9) for deaths that occurred before January 1, 1996 (~50% of all deaths) and according to the tenth revision (ICD-10) for deaths that occurred from January 1, 1996 onwards. CHD was defined as ICD-9 codes 410-414 or ICD-10 codes I20-I25.

Definition of the cohort

We used a case-cohort design for the present study. This means that the plasma tHcy, B-vitamins and creatinine concentrations were determined for all subjects (cases) that died of CHD during January 1987 and January 2000 (n=170) and a random sample (n=749), also called the *reference*- or *sub*-cohort, of the total Monitoring Project population. No stratification or matching was performed for the random sample that served as the source of information about the full cohort.

Laboratory measurements

During the physical examination, non-fasting venous blood samples were collected in EDTA tubes. To obtain plasma, the samples were centrifuged after a maximum storage period at room temperature of 3 hr. Non-fasting plasma total- and HDL cholesterol were enzymatically determined using a Boehringer test kit, within three weeks after collection. HDL cholesterol was determined after precipitation of the apo-B-containing lipoproteins with magnesiumphosphotungstate (28). The cholesterol levels were determined at the Central Clinical Chemistry Laboratory of the University Hospital Dijkzigt in Rotterdam, a reference laboratory that participated in the standardization program of the World Health Organization.

For the present study, additional laboratory analyses were carried out in plasma samples that were stored at -20°C for 10-14 years. The laboratory technicians were unaware of the case-status of the samples. The tHcy concentration, including the protein-bound and non-protein-bound fractions of homocysteine, was measured using high performance liquid chromatography with fluorescence detection, as described by Fiskerstrand (29) with some modifications (30). The within- and between-run coefficients of variation (CV) were 2.9% and 6.1%, respectively.

Plasma folate and vitamin B12 was determined with a commercial radio assay (Quantaphase II, B12/Folate radioassay, Bio-Rad Diagnostics Group) in the laboratory of Dr. Jacob Selhub of the Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA. For folate the within- and between- run CV's were 11.4 and 13.1%, respectively and for vitamin B12 5.1 and 7.6%, respectively. Plasma vitamin B6 (measured as pyridoxal 5' phosphate, i.e. PLP) was measured with a semi-automated fluorometric method by high-performance liquid chromatography, with a within- and between-run CV of respectively 5.7% and 9.7%. Plasma

creatinine was determined with a modified Jaffe method, with a within- and between- run CV of respectively 1.1% and 1.5%.

Data analyses

All statistical analyses were done with SAS statistical software (version 8.1) (SAS Institute Inc., Cary, North Carolina, USA). We only used subjects with complete information on plasma concentrations of tHcy, B-vitamins and creatinine, blood pressure, cholesterol level, smoking, history of cardiovascular diseases and diabetes: i.e. 154 CHD cases and 669 subjects from the reference cohort (n=821 in total, as 2 CHD cases belonged also to the reference cohort). Additionally, 89 subjects were excluded because they reported prevalent cardiovascular diseases at baseline. Thus, all analyses in this report are based on 732 subjects: 103 incident CHD cases and 630 reference subjects, as 1 CHD case belonged also to the reference cohort.

To determine the relation of the tHcy level with other variables, the reference cohort was divided into equal thirds (tertiles) according to the tHcy distribution. Analyses of covariance implemented in the SAS "PROC GLM" procedure were used to compute age, sex and study-center adjusted mean levels, or percentages, of variables by tertiles of the tHcy concentration. Trends over the tertiles were computed with linear regression analyses, modeling the tertiles as a continuous variable. For the concentrations of tHcy, B-vitamins and creatinine we present geometric means, as these variables were skewed towards higher values and we used logarithmic transformations to normalize their distributions.

To determine the relation between several variables and case-status, we computed mean, or percentage, values with analyses of covariance for cases versus the reference cohort, and tested the difference with "PROC GLM".

Cox's proportional-hazard analysis was used to estimate the relative risks (RRs) of CHD mortality in relation to tertiles of tHcy and plasma B-vitamin levels. Additional analyses with the independent variables as continuous variables were also performed. To calculate valid 95% confidence intervals (CIs) around the RRs in this case-cohort design (31,32) we used a SAS macro available at http://lib.stat.cmu.edu.general/robphreg. Interaction terms between our independent variables of interest (plasma levels of tHcy and B-vitamins) and age, sex, smoking, hypertension and cholesterol level, were evaluated. As we found a significant interaction between plasma folate and sex, we stratified our analyses for sex.

Variables that were included as possible confounders in multivariate models were age, study center (indicator variables for Maastricht and Doetinchem with Amsterdam as reference center), creatinine level, hypertension, smoking (no/yes), HDL cholesterol, total cholesterol, BMI, physical activity (indicator variables for regularly active and regularly strenuously active, with inactive subjects as reference), education (indicator variables for high and medium education, with low education as reference), and alcohol consumption (indicator variables, in men: >0 - ≤2

glasses/d and >2 glasses/d with abstainers as reference, in women: >0 - ≤1 glasses/d and >1 glasses/d, with abstainers as reference). As there were only 7 subjects (4 CHD cases) who had a history of diabetes after exclusion of subjects with cardiovascular diseases at baseline, we did not adjust for this variable.

Results

Characteristics of the cohort

Table 1 shows the age, sex and study-center adjusted mean levels or percentages of variables in the reference cohort according to the tertile distribution of the tHcy concentration. Variables that were inversely associated with the tHcy concentration were plasma levels of B-vitamins and B-vitamin supplement use. Variables that were positively associated with the tHcy concentration were creatinine level, diastolic blood pressure and prevalence of hypertension (borderline). Other lifestyle and CHD risk factors were not associated with the tHcy concentration.

Table 1 Age-, sex- and study center-adjusted mean levels of study variables according to tertiles (T) of the tHcy concentration in the reference cohort

Variable	Tertiles of tHcy			
	T1 (n=207)	T2 (n=206)	T3 (n=217)	P for trend
tHcy (µmol/L)	11.0	13.8	19.2	
Range	6.4-12.6	12.7-15.3	15.4-53.9	
Plasma folate (nmol/L	7.5	6.3	5.1	0.0001
Plasma PLP (nmol/L)	44.7	40.9	36.6	0.0001
Plasma vitamin B12 (pmol/L)	328.5	298.2	276.7	0.0001
Plasma creatinine (µmol/L)	102.0	104.7	109.3	0.0001
Systolic blood pressure (mm Hg)	119.3	120.4	121.4	0.13
Diastolic blood pressure (mm Hg)	74.9	75.6	76.9	0.04
Total cholesterol (mmol/L)	5.6	5.5	5.5	0.7
HDL cholesterol (mmol/L)	1.3	1.3	1.3	0.3
BMI (kg/m^2)	24.6	24.5	24.7	0.9
Alcohol (glasses/d)	1.2	1.4	1.0	0.2
Coffee (cups/d)	4.6	5.0	4.7	0.6
Smokers (%)	37	34	42	0.3
B-vitamin supplement users (%)	24	22	15	0.02
Hypertension (%)	13.5	17.9	19.9	0.07
Diabetes (%)	0.9	0.5	0.04	0.2
Inactive (%)	24	25	27	0.4
Low education (%)	68	57	64	0.3

Mean differences between CHD cases and non-cases

Table 2 shows the variables that were associated with case-status. Cases were older, were more likely to be male, and had higher tHcy and lower plasma folate and PLP concentrations. Furthermore, their CHD risk profile was unfavorable compared with non-cases. For example,

they had a higher blood pressure (systolic and diastolic) and total cholesterol level. Furthermore, their HDL cholesterol level was lower, and they were more likely to be smokers.

Relative risk of CHD

During a mean follow-up of 10.3 years, 78 men and 25 women died of CHD. Table 3 shows that there was a statistically non-significant positive association between the tHcy concentration and the incidence of CHD mortality in men as well as in women, after correction for age and study center. The relative risk (RR) of CHD mortality for those in the highest tertile (T3) of tHcy levels versus the lowest tertile (T1) was 1.32 (95% confidence interval (CI)=0.68-2.55) in men and 2.30 (95% CI=0.72-7.34) in women. After additional adjustment for CHD risk factors (HDL cholesterol, total cholesterol, hypertension, creatinine and smoking) the RR attenuated. As there was no statistically significant interaction between sex and the tHcy concentration on the RR of fatal CHD, we calculated the RR for each 5 µmol/L increase in tHcy concentration for men and women together. After adjustment for the variables of model 2 (table 3) and for sex, we found a RR of 1.03 (95% CI=0.83-1.29).

Table 2 Age-, sex- and study center-adjusted mean levels of study variables in CHD cases versus non-cases

Variables	Non-cases (n=630)	Fatal CHD cases (n=103)	P for difference
Age (years) (not adjusted for age)	41.5	51.6	0.0001
Males (%) (not adjusted for sex)	48	75	0.0001
tHcy (µmol/L)	14.4	15.4	0.04
Plasma folate (nmol/L)	6.3	5.7	0.03
Plasma PLP (nmol/L)	41.1	34.6	0.008
Plasma B12 (pmol/L)	302.5	283.7	0.1
Plasma creatinine (µmol/L)	106.1	106.8	0.6
Systolic blood pressure (mm Hg)	121.1	130.4	0.0001
Diastolic blood pressure (mm Hg)	76.4	82.1	0.0001
Total cholesterol (mmol/L)	5.6	6.0	0.0003
HDL cholesterol (mmol/L)	1.3	1.1	0.0001
Coffee (cups/d)	4.8	5.1	0.4
Alcohol (glasses/d)	1.3	1.4	0.4
BMI (kg/m^2)	24.7	26.7	0.0001
Smokers (%)	37	72	0.0001
B-vitamin supplement users (%)	20	23	0.5
Hypertension (%)	19	40	0.0001
History of diabetes (%)	0.5	4	0.003
Inactive (%)	25	24	0.8
Low education (%)	64	75	0.03

The plasma folate concentration was inversely associated with the risk of CHD mortality in women (P for trend 0.03) after correction for age and study center. There was no association in men. Additional correction for CHD risk factors did not materially change the protection of a high folate level for fatal CHD in women. The RR of CHD mortality for women in T3 of plasma folate versus T1 was 0.22 (95% CI=0.06-0.87). To check whether this effect was independent of the level of other B-vitamins and tHcy we also adjusted for plasma concentrations of tHcy,

PLP and vitamin B12, and vitamin B supplement use. This resulted in a RR for T2 versus T1 of 0.45 (95% CI=0.11-1.92) and a RR for T3 versus T1 of 0.22 (95% CI=0.04-1.32). Thus, the RR estimates did not change, however the confidence intervals widened. The adjusted (model 2, table 3) RR for each 5 nmol/L increase in plasma folate concentration was 0.16 (95% CI=0.03-0.78) in women.

Table 3 Relative risks (RR) and 95% confidence intervals (CI) of tertiles of the concentration of tHcy, and B-vitamins in relation to CHD mortality

	Men (n=379)				Women (n=353)			
	T1	T2	T3	P for trend	T1	T2	T3	P for trend
Mean[a] plasma tHcy (µmol/L)	11.4	14.5	20.1		10.6	13.3	18.4	
No. cases %	25 6.6%	19 5.0%	34 9.0%		5 1.4%	7 2.0%	13 3.7%	
RR model 1[b] 95% CI	1	0.63 0.31-1.31	1.32 0.68-2.55	0.4	1	1.48 0.42-5.23	2.30 0.72-7.34	0.2
RR model 2[c] 95% CI	1	0.78 0.31-1.93	1.14 0.50-2.61	0.7	1	1.31 0.28-6.05	2.04 0.48-8.62	0.3
Mean[a] plasma folate (nmol/L)	4.2	6.1	9.5		4.0	6.1	9.6	
No. cases %	25 6.6%	21 5.5%	32 8.4%		12 3.4%	8 2.3%	5 1.4%	
RR model 1[b] 95% CI	1	0.87 0.43-1.77	1.31 0.68-2.52	0.4	1	0.40 0.14-1.13	0.26 0.08-0.85	0.03
RR model 2[c] 95% CI	1	1.15 0.50-2.62	2.00 0.82-4.87	0.1	1	0.45 0.14-1.45	0.22 0.06-0.87	0.03
Mean[a] plasma PLP (nmol/L)	28.8	46.3	75.4		19.4	33.5	64.2	
No. cases %	45 11.9%	11 2.9%	22 5.8%		12 3.4%	5 1.4%	8 2.3%	
RR model 1[b] 95% CI	1	0.39 0.18-0.85	0.73 0.38-1.40	0.2	1	0.44 0.14-1.35	0.50 0.18-1.36	0.2
RR model 2[c] 95% CI	1	0.95 0.37-2.44	1.27 0.52-3.11	0.6	1	0.48 0.14-1.63	0.77 0.22-2.63	0.6
Mean[a] plasma vitamin B12 (pmol/L)	223.1	307.9	423.7		197.6	289.0	438.7	
No. cases %	30 7.9%	26 6.9%	22 5.8%		7 2.0%	11 3.1%	7 2.0%	
RR model 1[b] 95% CI	1	0.76 0.39-1.49	0.58 0.29-1.15	0.1	1	1.15 0.41-3.20	0.70 0.22-2.24	0.5
RR model 2[c] 95% CI	1	0.72 0.30-1.69	0.95 0.40-2.29	0.8	1	1.30 0.40-4.18	0.99 0.26-3.74	0.9

Explanation of abbreviations: a=Geometric mean; b=Adjusted for age and study center; c=Adjusted for age, study center, HDL cholesterol, total cholesterol, hypertension, creatinine and smoking.

We observed a statistically non-significant positive association between higher plasma folate levels and higher risk of CHD mortality in men, after multiple adjustment. As this was unexpected, we compared the characteristics of male cases in T3 with those in T1 and T2. Cases in T3 had a lower concentration of tHcy and total cholesterol, were less often smokers and had higher HDL cholesterol levels. Only their systolic and diastolic blood pressures were somewhat higher compared with men in T1. Thus, in general they were healthy cases, which may explain the higher RR after correction for CHD risk factors. The adjusted RR for each 5 nmol/L increase in folate concentration in men was 1.41 (95% CI=0.76-2.63).

We observed no statistically significant associations between plasma PLP and plasma vitamin B12 and incidence of CHD mortality in men as well as women. The adjusted RR for each 10 nmol/L increase in plasma PLP concentration in men and women was 1.03 (95% CI=0.99-1.07), and for each 50 pmol/L increase in vitamin B12 concentration the adjusted RR was 0.99 (95% CI=0.85-1.16).

Adjusting our multivariate models also for education, physical activity, alcohol consumption and body mass index did not lead to other conclusions. Additional exclusion of subjects who reported diabetes at baseline also did not materially change our results, but only affected the power of our analyses. For example, in women the RR for each 5 nmol/L increase in plasma folate concentration changed to 0.21 (95% CI=0.04-1.00) from 0.16 (95% CI=0.03-0.78).

Discussion

In this prospective study we found no statistically significant association between the plasma tHcy concentration and the incidence of CHD mortality. We found a significant protective effect of a higher plasma folate concentration for the risk of CHD mortality in women only. Plasma PLP and vitamin B12 concentrations were not associated with the incidence of CHD.

There are several methodological factors that might have contributed to an underestimation of any true effects. Firstly, due to the fact that the present study was not designed to measure the tHcy concentration, whole blood was not cooled nor centrifuged within 1 hr after drawing. This could have led to an increase in the tHcy concentration in the blood samples, which is ~0.6 µmol/L per hour, independently of baseline tHcy concentration (33). Secondly, our plasma samples were stored at -20°C for a maximum period of 14 years. For the plasma concentration of tHcy (34-36) and vitamin B12 and PLP (37) this was probably not a substantial problem. However, folate levels decline in a systematic manner (37). Nevertheless, our data on tHcy and folate concentrations were probably suitable to study associations, since the Spearman correlation and the regression coefficient for the relations between concentrations of plasma folate and tHcy, and between concentrations of plasma B12 and tHcy in the present study were

similar to those in our large cross-sectional study (33). In the latter study all plasma samples were centrifuged within 1 hr after drawing and were predominantly stored at –80°C for ~6 years (33). Finally, in the present study biochemical parameters were measured only once. Due to measurement errors and within-person fluctuations in blood parameters, a single measurement may underestimate the magnitude of the RR (38), referred to as regression dilution bias.

The number of fatal CHD cases was relatively small after exclusion of subjects who reported a history of cardiovascular diseases. Judging from the RRs of CHD mortality calculated in table 3 (T3 vs. T1 in men: 1.14, 95% CI=0.50-2.61; in women: 2.04, 95% CI=0.48-8.62) it might seem that we lacked statistical power to identify significant risk estimates. Yet, the RR calculated for each 5 µmol/L increment in tHcy concentration for men and women together (1.03, 95% CI=0.83-1.29) indicates that in this population the relation between the tHcy concentration and CHD mortality is weak.

One of the strengths of our study is that we adjusted for plasma creatinine, a marker for renal function. A high creatinine level indicates a decreased renal function, which leads to a decreased homocysteine clearance (39). Furthermore, a decreased renal function is associated with a higher risk of CHD (40). Therefore, omitting the correction for creatinine might lead to a higher risk estimate; without creatinine in our multivariate model the additive risk for each 5 µmol/L increase in tHcy concentration (for men and women together) doubled from 1.03 to 1.06 (95% CI=0.86-1.31). Another strength of our study is that we included men as well as women, which warrants the generalizability of our results to the whole adult population.

It seems unlikely that the previously described methodological factors are mainly responsible for the weak association between the tHcy concentration and CHD in the present study. The explanation for a weak association could lay in the proposed mechanism of action of an elevated tHcy level; it might be a short-term risk marker, which in the presence of other CHD risk factors promotes CHD (3). Evidence that an elevated tHcy level is a short-term marker is provided by data of the Physicians' Health Study. After 5 years of follow-up a significant increased risk of CHD was found in men with elevated tHcy levels (11), however, extending the follow-up to 7.5 years yielded a non-significant RR (27). Furthermore, in the same study population no significant association was observed between the tHcy concentration and the risk of angina pectoris after 9 years of follow-up (41). Two other studies also observed a stronger relation between the tHcy concentration and the risk of CHD mortality (23) and between the tHcy concentration and total cardiovascular disease mortality (14) during the first few years of follow-up.

Evidence that elevated tHcy levels may in particular promote CHD under conditions predisposing to CHD, comes from prospective studies which consistently show that an increased tHcy level is a risk factor for cardiovascular events (among which CHD) and total mortality in

subjects with an increased CHD risk (10,19,40,42-49). Furthermore, of the five prospective studies with elderly populations (average age >60 years) (7,10,13,14,23), only one (23) did not show a clear significant association between the tHcy concentration and CHD. As in elderly the chance of silent, pre-clinical, CHD is larger, this finding is in line with the hypothesis that an elevated tHcy concentration triggers an acute event in high-risk populations. Nevertheless, these results could also indicate that an elevated tHcy concentration is a marker of pre-clinical CHD.

A short-term harmful effect of an elevated tHcy concentration was not observed in our study population when we looked at the risk of CHD mortality in the first 5 years of follow-up (data not shown). Yet our data supported the suggestion that raised tHcy levels might trigger an event in high-risk subjects, as the RR of CHD mortality per 5 µmol/L increment in tHcy concentration in subjects with cardiovascular diseases at baseline was 1.58 (95% CI=0.81-3.09).

In summary, the fact that we studied relatively young subjects, free of baseline CHD could be an explanation for the observed weak association between the tHcy concentration and CHD. This result, although weak, is generally in line with other prospective studies with subjects not selected for their risk of CHD (7-23). Based on most of these studies (7-9,11-18,21-23) Ueland *et al* (50) recently calculated a pooled odds ratio for each 5 µmol/L increase in tHcy concentration of 1.20 (95% CI=1.14-1.25). Our estimate (1.03, 95% CI=0.83-1.29) revealed a much lower RR for each 5 µmol/L increase in tHcy concentration, but the confidence intervals of both estimates overlap.

We found that high folate concentrations were associated with a significant decrease in RR of CHD mortality in women only, which is in line with the results of Morrison *et al* (24). Other studies that investigated plasma folate concentration in relation to CHD observed inverse relations in men as well as women (25), or in men only (26). Two studies did not find significant associations (16,27), although the risk estimate was compatible with the hypothesis that high folate levels might contribute to the protection of CHD. In addition, other prospective studies that investigated the relation between folate status and all cardiovascular diseases as endpoint, showed a beneficial effect of higher folate levels (51-53). Thus, it is not unlikely that folate might have a protective role in the development of CHD. In our study, this protective effect in women was (at least partly), independent of the tHcy concentration.

The concentration of the other two B-vitamins that were investigated in this study, plasma PLP and vitamin B12 did not show a relation with CHD. Evidence from previous prospective studies is not entirely consistent but suggests that the PLP concentration may be inversely associated with CHD (16), although not always statistically significant (27). In the present study, the simple adjusted models suggested a protective effect of higher PLP levels on CHD

risk. However, this disappeared after correction for traditional CHD risk factors. The null finding with the plasma vitamin B12 concentration agrees with that found in the ARIC study (16).

In conclusion, we found that elevated tHcy levels are not a major risk factor for CHD in a population sample of Dutch subjects below the age of 60 years and free of cardiovascular diseases at baseline. On the other hand, high plasma folate concentrations were associated with a lower CHD risk, in women only. Randomized trials are needed to clarify the complex relations between plasma concentrations of tHcy and B-vitamins, and CHD (6).

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10

General Discussion

Introduction

The main objectives of this thesis were to investigate possible determinants of plasma total homocysteine (tHcy) concentrations, and to quantify the association between tHcy concentrations and the risk of coronary heart disease (CHD) mortality. Both the determinants and the association with CHD were explored in general Dutch adult populations.

This general discussion starts with an evaluation of the utilized study populations. Thereafter, the measurement of main variables and methodological issues accompanying the measurement of these variables are discussed. Then our main findings with respect to tHcy-determinants are summarized and evaluated in the context of other studies and recent findings. Subsequently, the association between the tHcy concentration and CHD is discussed in light of the mechanism by which elevated tHcy concentrations might increase the risk of CHD, taking the ongoing discussion on whether or not the tHcy concentration is causally related to CHD into consideration. Consequently, some directions for future research are given, finishing with implications of our results for prevention and treatment.

Evaluation of the study populations

The results described in this thesis are mainly based on data of two large monitoring studies. The Monitoring Project on Risk Factors and Health in the Netherlands (MORGEN study) (1) was used for studying the tHcy-determinants cross-sectionally. The data from the Monitoring Project on Cardiovascular Disease Risk Factors (2) were the basis for our prospective study in which we quantified the relation between the tHcy concentration and the risk of CHD mortality.

Selective participation and loss to follow up

Full participation in the monitoring studies required completing extensive self-administered questionnaires and being physically examined at one of the study centers. This considerable effort might have contributed to the relatively low overall response rate (i.e. ~50%). However, about 10% of the non-response could be ascribed to errors in the municipal population registration and to the fact that people had moved since the address data were obtained (2). Generally, participants (respondents) of extensive health surveys are more health conscious than non-respondents are. Non-response surveys of both monitoring studies indicated that non-respondents were more likely to be male, and to be smokers (2). This selective response did probably not affect the results of the cross-sectional study (discussed in chapter 3). With regard to the prospective study, the number of fatal CHD cases was somewhat lower than expected based on the number of persons examined (~36,000), the age and sex distribution of the respondents and the mean follow-up; 170 vs. 180 CHD deaths. It seems unlikely that this lower

number was due to relatively healthier participants in our study compared with the general Dutch population. A more plausible explanation is that medical treatment has improved over the years, which leads to longer survival of CHD patients. Loss to follow-up was not a major concern since this was only 0.02% (chapter 9).

Study size and study design aspects

The cross-sectional study population was a large (n=3025) age and sex stratified random sample of the MORGEN study. This enabled us to study the effect of various tHcy-determinants in a balanced manner for both men and women in different age categories. However, further stratification (e.g. with respect to MTHFR 677 C>T genotype), reduced statistical power.

A case-cohort design was used for the prospective study. This is a design developed to (cost-)efficiently use the data of a full cohort, comparable to a nested case-control design. Participants of the Monitoring Project on Cardiovascular Disease Risk Factors were the source population for our case-cohort study. Of all these subjects data were collected during 1987-1991 (baseline). A random sample was drawn from the full cohort after an average follow-up of 10.3 years. This random sample is called the *sub*-cohort. The sub-cohort was not stratified and included also a few CHD cases. The remaining majority of CHD cases that were not part of the sub-cohort were added to the sub-cohort. In other words, the case-cohort population consists of all cases that occurred during follow-up in the full cohort and the non-cases included in the sub-cohort.

The major advantage of the case-cohort design above the nested case-control design is that the sub-cohort can provide information on the full cohort (3). Thus, due to information on person-years at risk of exposed and non-exposed subjects, the same epidemiological incidence measures can be calculated as in a full cohort study. To achieve precise measures, the size of the sub-cohort should be about four-fold or more of the number of cases (4). Our sample was about six-fold larger than the number of cases.

The specific features of a case-cohort study compared with a full cohort and a nested case-control study are summarized in box 1.

Box 1 The case-cohort design

A case-cohort study efficiently uses information of a full cohort study. For the full cohort information of all subjects is collected at baseline. After follow-up, a random sample from the full cohort is drawn (sub-cohort), including non-cases and cases. All remaining cases that were not part of this random sample are added (3).

Schematically the data used in a full cohort study, a nested case-control study, and a case-cohort study can be presented as follows (adapted with permission from (3)):

Exposure	CHD deaths	Non-CHD deaths	Total number	Person years
	Full cohort design			
exposed	A	B	A+B	PY1
non-exposed	C	D	C+D	PY0
total	A+C	B+D	N	PY
	Nested case-control design			
exposed	A	b	?	?
non-exposed	C	d	?	?
total	A+C	b+d	?	?
	Case-cohort design			
			random sample from cohort	
exposed	A	b'	a' + b'	py1
non-exposed	C	d'	c' + d'	py0
total	A+C		n	py

Capitals: numbers in the full cohort, small print: samples from the cohort
a', b', c' and d': these subjects are the random sample of the full cohort. In the statistical analysis these subjects are the source of information on person-years of (non-) exposure of the full cohort.

The RELATIVE RISK (RR) can be estimated both with the nested case-control design and with the case-cohort design:
In the full cohort study, this is a ratio of the ABSOLUTE RISK in exposed and non-exposed: [A/A+C]/ [B/B+D];
In the nested case-control study, this is the exposure ODDS RATIO: [Ad/bC]. Note that this ratio estimates the disease odds ratio of the cohort, and provided that A and C are small (rare disease assumption), it approaches the RR in the cohort;
In the case-cohort study this is estimated by the case-cohort ODDS RATIO: [A/(a'+b')]/[C/(c'+d')], which is an estimate of the RR in the cohort, whether the outcome is rare or not.

In contrast to the nested case-control design, a case-cohort design has information on the person-years exposed or non-exposed. Therefore, several additional measures can be calculated which reflect those in the full cohort:
INCIDENCE DENSITY or incidence rate, which is the number of CHD cases during a given period of time as a proportion of the total person time at risk in the population:
among exposed=a'/py1
among non-exposed=c'/py0
INCIDENCE DENSITY RATIO, which is a more valid measure of the RR in studies with a long follow-up or in the presence of competing mortality: [a'/py1]/[c'/py0] (4).

If the sample fraction, i.e. f=n/N, is known, the corresponding numbers or person-years in the cohort can be estimated, for example the estimate of A+B equals: (a'+b')/f. With these calculations absolute cumulative incidence and absolute incidence densities can be calculated (4)

The sub-cohort can be used as a reference sample for different outcomes (5), which is also an advantage above the nested case-cohort design. A draw back of the case-cohort design lies in the fact that the standard error of the RR estimates in multivariate analyses is invalid due to dependency in the data, existing between the case-group and the referent group. However, recently a Cox's proportional-hazard method that calculates valid standard errors for this type of study design is available for SAS-software as a macro available from the internet http://lib.stat.cmu.edu.general/robphreg.

Measurement of main variables

Errors in the measurement of variables lead to misclassification that may affect the validity of study results. The result of measurement error can be differential (dependent on the values of other variables) or non-differential (independent of the values of other variables). Non-differential random misclassification may weaken the effect estimates. Non-differential systematic misclassification will not change the effect estimates. The specific effect of differential misclassification on the results is difficult to determine; hence this type of error is of greater concern because it can give spurious associations. In the following section several issues that might have contributed to measurement errors in this thesis will be evaluated.

Conditions prior to the measurement of plasma tHcy and B-vitamins concentrations

Table 1 summarizes the recommendations regarding collecting, handling and storing blood samples for the measurements of tHcy and B-vitamin levels. Furthermore, it shows to what extent we fulfilled these recommendations and what the effect might have been on our results.

Generally it is recommended to collect fasting blood samples for the measurement of the tHcy concentration (6). However, for the monitoring surveys that were used in this thesis, it was not feasible to obtain fasting blood samples. For the cross-sectional study we had information on the time of the last taken meal or drink. The tHcy concentration of fasting samples was on average 0.7 µmol/L higher than in non-fasting samples (see chapter 3). Yet, the number of fasted subjects was very low (3%), thus, substantial bias of our effect estimates is unlikely.

The tHcy concentration of blood collected in sitting position is lower (maximally 30%), than that of blood collected after a supine (horizontal) rest (7). As blood collection in our studies was standardized to sitting, this should be taken into account when horizontally collected samples (e.g. of bedridden patients) are compared with our values.

The tHcy concentration increases in whole blood when left at room temperature (8,9). This is probably due to a continuous synthesis and export of homocysteine from blood cells (9,10). To overcome this increase in tHcy concentration, one should centrifuge or cool blood immediately after drawing. The samples of the cross-sectional study were centrifuged within 1 h, but the samples of the prospective study were stored at room temperature for a maximum of 3 h.

Table 1 Recommendations regarding collecting, handling and storing blood samples for the measurements of tHcy and B-vitamin concentrations

Recommendation	Recommendation fulfilled in:		Effect on the tHcy concentration if not handled according to the recommendations	Effect on B-vitamin concentrations if not handled according to the recommendations	Type of measurement error
	Cross-sectional study?	Prospective study?			
Collecting blood: subjects should be fasting to reduce effect of last eaten meal	no: samples were non-fasting, but information was collected on time of last taken meal	no: samples were non-fasting, no information was collected on time of last taken meal	tHcy concentration of fasting samples were 0.7 µmol/L higher compared with non-fasting samples (chapter 3)	no effect of fasting (data not presented)	non- differential
Handling blood: **only for tHcy concentration;** centrifugation within 1 hr or direct cooling of whole blood	yes: random sample selected from subjects of which blood was centrifuged within 1 h	no: maximum storage at room temperature for 3 h	0.6 µmol/L increase in tHcy/h, independent of baseline value (chapter 3)	not relevant for B-vitamins	non- differential
Storing plasma: at or below -80°C	yes, partly: storage at –20°C for maximum of 17 months -80°C for maximum 51 months	no: storage at -20°C for 14 years	probably negligible as the tHcy concentration remains stable at room temperature for 2 weeks (11), and stable at -20°C for at least 2 years (8,11,12)	B12 and B6 remain stable for at least 4 years, folate declines systematically independent of baseline value (13)	non- differential

In chapter 3 we showed that the increase in tHcy concentration is independent of baseline tHcy levels. Thus, when all whole blood samples are left at room temperature for the same amount of time, the increase in tHcy concentration is similar for all samples, thereby leaving the ranking of subjects according to their tHcy level intact. However, the duration of storage at room temperature of the samples from the prospective study varied; some might have been centrifuged within 1 h, whereas others will have been centrifuged after 3 h. This will have introduced some random error. To evaluate this error we compared the age- and sex-specific mean tHcy concentrations of the prospective study with those of the cross-sectional study (in which all samples were centrifuged within 1 h). This showed that on average the values of the prospective study were 1 µmol/L higher. The maximal standard deviation (SD) of this increase is estimated to be no larger than 1 µmol/L. This SD is small compared with the SD of the tHcy concentrations of the case-cohort population, i.e.: 5.6 µmol/L (n=732). Therefore, the extraneous variation introduced by the increase will only have had a small diluting effect on the association between the tHcy concentration and CHD mortality.

Misclassification due to altered concentrations as a result of prolonged storage of the plasma samples, again is mostly restricted to the prospective study. Storage at relatively high

temperatures (i.e. -20°C) is especially deleterious for the concentration of folate, that declines in a systematic manner (13), possibly because some of the folates are destroyed through oxidation (14). This could explain why the plasma folate concentrations were on average somewhat lower in the prospective study compared with the cross-sectional study (6.7 vs. 7.4 nmol/L). Yet, the storage conditions did not affect the ranking of subjects, as the Spearman correlation between the plasma folate and tHcy concentration in the prospective study was equal to that in the cross-sectional study (i.e. r=-0.4). Thus, the absolute levels of the plasma folate concentration need to be interpreted with caution, yet the data were suitable to study associations because the ranking of subjects remained intact.

Absence of a gold standard

For the determination of tHcy or B-vitamin concentrations in plasma no ultimate reference method is available. Of all methods to determine the tHcy concentration, i.e. capillary electrophoresis, immunoassays, gas-liquid chromatography and various methods of high performance liquid chromatography (HPLC) (15), the gas chromatography mass spectrometry assay is sometimes used to establish the relative validity of another method because it is sensitive and specific (16). For the measurements of the concentration of B-vitamins the number of available methods is also substantial.

Appreciating that each laboratory has its own adaptations for these methods, the variation in methods is large. This is reflected in the considerable differences in measured concentrations of e.g. tHcy (i.e. 6-15%) (17-20) and folate (i.e. 27.6%) (21) in one sample determined in several laboratories. In some cases the between-laboratory differences exceeds the between-method variation (20).

Due to the inter-laboratory differences, concentrations measured in one laboratory cannot be reliably compared with those determined in another laboratory without evaluation of inter-laboratory differences (see also chapter 3). This severely complicates the establishment of normal levels, and as a consequence of this, also of deficient (in case of vitamins) and elevated (in case of tHcy) levels. Certified reference material is urgently needed to improve inter-method and inter-laboratory agreement (16). An external quality control program, analogous to the cholesterol standardization program of the World Health Organization (WHO), is needed to supervise and to maintain standardization.

In the absence of a gold standard, absolute validity can not be assessed. However, the incorporation of reference plasma samples in an analytical run allows the calculation of the precision, i.e. the reproducibility of a measurement. For the cross-sectional and the prospective study we used the most common method to measure the tHcy concentration: high performance liquid chromatography (HPLC) with fluorometric detection. The within (<3.2%) and between (<8.6%) run coefficients of variation (CV) of this method indicate that there is close agreement

between successive measurements of the same sample and the results obtained with the same method under different conditions. In the cross-sectional study the folate and vitamin B12 concentrations were determined microbiologically, whereas for the prospective study a radio assay was applied. The coefficients of variation with the radioassay were higher compared with those of the microbiological assay, but they were acceptable (CV-within <11%, CV-between <13%). The plasma vitamin B6 concentration (measured as pyridoxal 5' phosphate, i.e. PLP) was measured with a HPLC method with satisfying within- and between-run CVs (5.7% and 9.7%, respectively).

Data on habitual dietary intake was only used in the cross-sectional study. For this purpose we used a semi-quantitative food-frequency questionnaire. The relative validity was assessed in 63 men and 58 women by calculating the Spearman correlation between the food-frequency questionnaires and the average of 12 24-h dietary recalls (22). Of the main contributors to the B-vitamin intake, the relative validity was somewhat low for vegetables (r=0.38 in men and r=0.31 in women), and for meat, but only in men (r=0.47). In women the relative validity of reported meat consumption was high (r=0.70). The relative validity of other foods groups mainly contributing to the B-vitamin intake (bread, potatoes, milk and milk products) and of alcoholic and non-alcoholic drinks, ranged between 0.58 and 0.87. Overall, the relative validity of this questionnaire was generally sufficient. The low relative validity for vegetables was also observed for other questionnaires (reviewed in (22)).

Once an estimate of habitual intake is acquired, this needs to be translated into nutrients. For this translation, food composition tables are necessary. The use of such tables introduces measurement errors at various levels. Errors may result from the assumption that the nutrient content of a specific food is constant, which in reality may vary considerably depending on e.g. sunlight, soil, and type of breed. Furthermore, when the foods sampled for the analytical determination do not represent those commonly eaten, this leads to errors. Finally, errors are introduced due to inaccuracy of the analytical data, missing data, and incorrect use of data from other food composition tables (23).

For the studies described in chapter 4 and 5, we used the most recent data available for foods commonly eaten in the Netherlands. Dutch data for folate were not available at the time our study started, but they were provided by Konings *et al* who measured folates in foods with an improved HPLC method (24,25). The estimated folate intake with this advanced method was about 30% lower compared with an estimate based on the British food composition table (26). The British table was used for comparison due to its completeness and the fact that all food samples were analyzed using the same method. Despite differences in absolute intake level, the ranking of subjects according to their intake was similar for the two used food composition tables; the Spearman correlation between folate intake calculated with the British table and with the recent Dutch values was high (i.e. r=0.88).

Intra-individual variation

Intra-individual variation in plasma parameters and dietary intake may also introduce measurement errors. Concerning plasma parameters, this variation is due to errors in the measurement, but also to real hour-to-hour or day-to-day biological fluctuations. Using an average of repeated measurements will reduce this type of non-differential error, but in our study we only had single measurements. From the literature it is known that the intra-individual coefficient of variation in tHcy concentration is ~9% day-to-day (7), ~7-8% over a month (27,28) and 9% over a year with little seasonal variation (29). This indicates that a single measurement reasonably reflects a persons average tHcy level. Furthermore, this level of intra-individual variation is comparable with that of total cholesterol and systolic blood pressure (30). To our knowledge, data on the intra-individual variation in levels of B-vitamins are not available.

Intra-individual variation in dietary intake may result in an attenuation of an association when the reported dietary intake does not represent the long-term habitual dietary intake. The reproducibility of reported dietary intake with the food-frequency questionnaire used in the cross-sectional study was evaluated in the previously described validation study (22). This was done by calculating the Spearman correlation coefficient between food groups reported in 2 questionnaires completed 12 months apart. Of the food groups that predominantly contributed to the intake of our main nutrients, the correlation for vegetables was 0.65 for women and 0.76 for men. For the other food groups (bread, potatoes, milk and milk products, meat, alcoholic and non-alcoholic beverages) it ranged between 0.68 (for meat in men) and 0.92 (for alcoholic beverages in women) (22). This indicates that the reported dietary intake did not change considerably over 1 year.

Although a sufficient reproducibility excludes a large effect of random errors in the reporting of food intake, it can not exclude conscious or unconscious under- or over-reporting of habitual food intake. Alcohol consumption is an item that is frequently under-reported, whereas vegetable and fruit intakes are often over-reported. When this happens systematically, the ranking of subjects remains the same. Consequently this will not have an effect on measures of association. However, when reporting depends on a persons' risk profile (like body mass index-dependent under-reporting (31)), this leads to differential misclassification of subjects. Differential misclassification can either overestimate an association (when high-risk persons exaggerate their unhealthy lifestyle) or underestimate an association (when high-risk persons deny their unhealthy lifestyle). Energy adjustments, like we applied for all nutrients, may reduce some measurement error due to reporting errors (32).

Multicollinearity between dietary variables

To assess an independent effect of intake of folate and vitamin B2, B6 and B12 on tHcy concentrations, we corrected the associations for intake of the other three B-vitamins and

methionine. Because these nutrients are frequently present in the same types of food, they are highly inter-correlated. This raises the problem of multicollinearity in multivariate regression models. For example, folate intake is highly correlated with vitamin B6 intake (Spearman r=0.8). Suppose a model in which the tHcy concentration is predicted by folate intake with a simultaneous correction for vitamin B6 intake. Due to the high correlation between folate and vitamin B6 intake, the degree in which folate intake can vary, is largely reduced by the fact that the level of vitamin B6 intake is statistically kept constant. Fortunately, energy adjustment largely reduced the inter-correlation of the nutrients from between 0.44-0.82 to 0.12-0.67. With the energy adjusted variables we evaluated whether we experienced a reduced variance in our models. This was done by calculating the variance inflation factor (VIF). For none of our models the VIF exceeded 2.4, whereas multicollinearity is to be concerned when the VIF is larger than 10.0 (33).

Remaining to the above-mentioned example, we may conclude that multicollinearity probably did not hamper the adjustment for the intake of other B-vitamins in the relation between folate intake and the tHcy concentration. This makes it less likely that the observed relation between folate intake and the tHcy concentration is in fact an underlying relation between e.g. vitamin B6 intake and the tHcy concentration. Moreover, the simultaneous correction for vitamin B2, B6, B12 and methionine intake while studying the relation between folate intake and the tHcy concentration will have reduced the chance of *residual confounding*. Residual confounding occurs if one cannot properly account for factors that are associated with the outcome (in this case the tHcy concentration) and the exposure (in this case folate intake).

Measurement of physical activity, smoking, biological CHD risk factors and genetic background

Physical activity is difficult to quantify, as it comprises activity during leisure time, work, study, household activities and commuting (e.g. cycling to work). Depending on the questionnaires used in the studies we distinguished 4 levels of activity (cross-sectional study) and 3 levels of activity (prospective study). Both classifications were positively associated with HDL cholesterol (34). As this is a recognized response to increased exercise levels (35,36), physical activity was probably ranked in a reasonably valid manner. Furthermore, the reproducibility and relative validity of a simplified version of the physical activity questionnaire used in the cross-sectional study was suitable for ranking subjects according to their level of physical activity (37).

In several chapters we used information on smoking habits, either to evaluate whether it interacted with other variables (chapter 4), to study the independent effect on tHcy concentrations (chapter 6), or to adjust for it as a confounder (chapter 5, 7-9). When classifying subjects into never, ex, light, moderate and heavy smokers, misclassification can occur at the

level of the number of cigarettes smoked per day. This classification was only used in chapter 6. For the other chapters we used a dichotomous variable indication whether a subject is a current smoker or not. The chance of recall bias for this crude variable is likely to be smaller. On the other hand, for those associations in which smoking is a strong confounder, residual confounding due to the lack of detailed smoking habits may be a problem. Fortunately, in none of our analyses smoking was strongly associated with our explanatory variables.

Total and HDL cholesterol levels were measured in fresh blood samples in a standardized reference laboratory, which largely decreases the chance of measurement errors. Yet, as we only had a measurement at one point in time, a dilution of effects can occur due to intra-individual variation (see previous section). Measuring blood pressure twice and using the average in the analyses reduced short-term intra-individual variation in systolic and diastolic blood pressure. For the other measurements, like waist circumference, weight and height, no special procedures other than standardized protocols were applied to reduce measurement errors that were likely to be small.

The outcome of genetic mutation analyses is, compared with the determination of e.g. an exact concentration, relatively simple since only three readings are possible (i.e. homozygous mutant, heterozygous, and homozygous normal). As a computerized reading, which is not influenced by personal interpretation, was not available for this genotype we designed a protocol to reduce reading and coding errors. Since our genotype distribution was in Hardy-Weinberg equilibrium and was similar to those reported in other Western-European studies (38,39), misclassification did not seriously affect the prevalence of the genotypes.

Measuring CHD mortality

To survey which subjects of our cohort had died between the start of the study (1987) and the censoring date (01-01-2000) we linked the unique personal identification numbers of our participants with data of municipal registries. Data on the date of death from these registries are known to be valid. When subjects could not be linked, the reason was investigated, resulting in a very low loss-to-follow-up (i.e. 0.02%). Subsequently, the cause of death was obtained from Statistics Netherlands. Coding of the causes of death could have involved some measurement error, which may have weakened any true associations. Nevertheless, the mortality of all types of cardiovascular diseases in this project was the same as in earlier monitoring cohorts in the Netherlands (40).

Discussion of main findings: determinants of homocysteine

Table 2 summarizes the findings on tHcy-determinants described in this thesis. In short, men and older subjects had higher tHcy levels. Folate intake was the only B-vitamin inversely associated with the tHcy concentration, showing a stronger relation in male non-drinkers and male smokers.

Table 2 Summary of main findings with respect to tHcy-determinants

Potential determinant	Association?	Effect on tHcy concentration	Ch
sex	yes	1.3 µmol/L higher tHcy in Γ vs. E	3
age	yes, E only	1.3 µmol/L higher tHcy in E 60-65 vs. E 20-29 years old	3
folate intake	yes	2.2 µmol/L higher tHcy in Q1 vs. Q5 in Γ; 1.3 µmol/L higher tHcy in Q1 vs. Q5 in E	4
vitamin B2 intake	no		4
vitamin B6 intake	no		4
vitamin B12 intake	no		4
interaction *folate intake ´ alcohol use*	yes, Γ only	3.4 µmol/L higher tHcy in Q1 vs. Q5 in non-drinking Γ; 1.8 µmol/L higher tHcy in Q1 vs. Q5 in Γ drinking ≤2 glasses/d; 1.4 µmol/L higher tHcy in Q1 vs. Q5 in Γ drinking >2 glasses/d	4,6
interaction *folate intake ´ smoking*	yes, Γ only	2.8 µmol/L higher tHcy in Q1 vs. Q5 in smoking Γ; 1.6 µmol/L higher tHcy in Q1 vs. Q5 in non-smoking Γ	4,6
MTHFR C677T polymorphism	yes	4.2 µmol/L higher tHcy in TT vs. CC genotype	5
interaction *folate intake ´ MTHFR C677T* polymorphism	yes	1.4 µmol/L higher tHcy in Q1 vs. Q5 in CC and CT subjects; 4.1 µmol/L higher tHcy in Q1 vs. Q5 in TT subjects	5
interaction *plasma folate ´ MTHFR C677T* polymorphism	yes	2.3 µmol/L higher tHcy in Q1 vs. Q5 in CC subjects; 3.6 µmol/L higher tHcy in Q1 vs. Q5 in CT subjects; 8.3 µmol/L higher tHcy in Q1 vs. Q5 in TT subjects	5
coffee consumption	yes	1.4 µmol/L lower tHcy in 0 vs. >6 cups of coffee/d in Γ; 1.1 µmol/L lower tHcy in 0 vs. >6 cups of coffee/d in E	6
tea consumption	no		6
smoking	yes, E only	0.8 µmol/L lower tHcy in non-smoking vs. E smoking ≥20 cigarettes/d	6
physical activity	yes, E only	0.6 µmol/L higher tHcy in heavily active vs. sedentary E	6
alcohol consumption	yes, Γ only	folate intake below median: 1.7 µmol/L higher tHcy in Γ drinking 0 vs. >2 glasses of alcohol/d; folate intake above median: 0.8 µmol/L higher tHcy in Γ drinking 0 vs. >2 glasses of alcohol/d	6
beer	yes, Γ only	1.3 µmol/L higher tHcy in Γ abstaining from beer vs. Γ in highest beer tertile (average 3.7 glasses of beer/d)	7
wine	no		7
spirits	no		7
total cholesterol	yes, E only	0.6 µmol/L lower tHcy in Q1 (≤4.47 mmol/L) vs. Q4 (>5.86 mmol/L) of total cholesterol in E	8
HDL cholesterol	yes, E only	1.0 µmol/L higher tHcy in Q1 (≤1.05 mmol/L) vs. Q4 (>1.52 mmol/L) in HDL cholesterol in E	8
waist circumference	no		8
diastolic blood pressure	no		8
systolic blood pressure	no		8

Explanation of abbreviations: Ch=Chapter in this thesis, Γ=men; E=women; Q1=first quartile or first quintile (depending of whether it is compared with Q4 or Q5), Q4=highest quartile, Q5=highest quintile, T1=first tertile, T3=highest tertile.

The 677 C>T polymorphism in the gene encoding for MTHFR was associated with higher tHcy levels. In addition, the relations between folate intake → plasma folate concentration, plasma folate concentration → tHcy concentration and folate intake → tHcy concentration were different for the three genotypes (i.e. CC=homozygous normal, CT=heterozygous, TT=homozygous mutant). The relations between the plasma concentration of folate and tHcy, and between folate intake and tHcy concentration were inversely for all genotypes and the steepest inverse slope was observed in TT subjects. The relation between folate intake and plasma folate was positive for all genotypes and strongest in CT subjects.

Of the lifestyle factors, coffee consumption was associated with higher tHcy levels in both men and women. Furthermore, smoking and physical activity were positively associated with the tHcy concentration in women, and alcohol consumption was inversely associated with the tHcy concentration in men. This inverse association was probably due to beer consumption.

Of the biological CHD risk factors, we only found weak associations in women: inverse between HDL cholesterol and the tHcy concentration, positive between total cholesterol and the tHcy concentration.

Age and sex

Consistent with other studies we found that men had higher tHcy levels than women (chapter 3). Some researchers have postulated that this difference between men and women is due to muscle mass (41). Creatinine is an indicator of muscle mass. The data of the prospective study (sub-cohort) indeed showed that after creatinine-adjustment there was no longer a relevant difference in the tHcy concentration between men and women (data not shown).

Age was positively associated with the tHcy concentration, which was only significant in women. We could not ascribe this relation to the occurrence of the menopause. This finding is in line with several other investigations (42,43), yet there is evidence that pre-menopausal women handle a methionine load more efficiently than post-menopausal women (44-46).

B-vitamin intake

Four B-vitamins are involved in homocysteine metabolism: folate, vitamin B2, B6 and B12. In our population, vitamin B2, B6 and B12 intake were not associated with tHcy levels after correction for intake of methionine and the other B-vitamins (including folate). We, and recently also others (47,48), showed that a higher dietary folate intake is associated with a lower tHcy level in adults, independently of other dietary and lifestyle factors (chapter 4). These results complement those found in observational studies on dietary folate intake and the tHcy concentration in middle aged (49,50) and elderly subjects (50-53). The fact that folate is the most important dietary determinant of tHcy levels, is in line with its metabolic role. Folate is used as a substrate: it donates the methyl-group for the conversion of homocysteine to

methionine (see figure 1, General Introduction). On the contrary, vitamin B2, B6 and B12 are not utilized when homocysteine is metabolized; they function as co-factors of enzymes in homocysteine metabolism.

Table 3 Folate intake: recommended, actual and desired intake with regard to tHcy levels in the Netherlands. Comparison between available data in 1997 and in 2001

1997				**2001**			
	men	women	reference		men	women	reference
Recommended folate intake				Recommended folate intake			
	200-300 µg/d	200-300 µg/d	chapter 2	remained the same as in 1997			
Actual folate intake (mean) calculated with the British food composition table (26):				Actual folate intake (mean) calculated with the Dutch food composition table (25):			
DNFCS	293 µg/d	238 µg/d	(55) chapter 2	DNFCS	215 µg/d	173 µg/d	(25)
				MORGEN study	239 µg/d	192 µg/d	chapter 4
Desired folate intake				Desired folate intake			
	>350 µg/d	>350 µg/d	chapter 2	MORGEN study, all	>300 µg/d	>300 µg/d	chapter 4
				MORGEN study, MTHFR 677 TT (10% of the population)	>>300 µg/d	>>300 µg/d	chapter 5

Explanations of abbreviations: DNFCS=second Dutch National Food Consumption Survey, MTHFR 677 TT=homozygous mutant.

Table 3 summarizes the main results with regard to folate intake. Between 1997 and 2001 the Dutch health authorities have not changed the dietary recommendations for folate. The only health authority that changed the dietary recommendation for folate from 200 to 400 µg/d, using data on the tHcy concentration as a functional parameter of the folate status, was the USA Food and Nutrition Board (54).

Due to the lack of Dutch data before 2001 (25), folate intake in the second Dutch National Food Consumption Survey (DNFCS) performed in 1992, was calculated with data mainly from the British food composition table (55). Recently, a validated HPLC method was developed with which the folate content of a large set of commonly eaten Dutch foods was measured (24). With the new data the folate intake in the second DNFCS was recalculated. The average folate intake was ~27% lower than calculated with the British food table. For the calculation of the folate intake in our cross-sectional study (chapter 4) we also used the new food composition data (25), and the average intake was similar to the estimate in the DNFCS.

A desired folate intake level was defined as the folate intake level associated with an optimally low tHcy concentration in light of the continuous relation between the tHcy concentration and the risk of CHD. In chapter 4, we showed that the relation between folate intake and the tHcy concentration decreased continuously across the measured folate intake

range: 140-300 µg/d, i.e. the average levels in first and last decile of the folate intake distribution. The tHcy concentration did not reach a low-plateau value within this range. Thus, we concluded that a desired folate intake level lies above 300 µg/d.

In chapter 5 we investigated the effect of the MTHFR 677 C>T polymorphism on folate requirements in the same population. The MTHFR enzyme produces 5-methyl-tetrahydrofolate, which is the main circulating form of folate used for the conversion of homocysteine to methionine. The homozygous mutant (TT) form of this polymorphism is especially associated with a reduction in specific enzyme activity (<50%) (56). The reduced activity of the enzyme was reflected in the higher folate intake requirements of TT subjects. At the highest folate intake levels, CC and CT subjects had a comparably low tHcy level (CC: 12.4 µmol/L and CT: 13.1 µmol/L). However, at this high folate intake level, TT subjects still had a high tHcy concentration (15.7 µmol/L). With these data we estimated that the desired folate intake of TT subjects lies at least 10% above the level for CT and CC subjects.

In summary, the desired folate intake estimated with available data in 1997 (>350 µg/d) is supported by our cross-sectional data. Using the improved food folate data resulted in a lower estimated folate intake than previously assumed, enlarging the gap between actual and desired folate intake.

Lifestyle factors

Along with findings of most (47,53,57-59), but not all (48,50,60), observational studies coffee consumption was positively associated with the tHcy concentration in both men and women (chapter 6). Recent intervention trials have shown that this effect of coffee is causal (61-63). Caffeine might be the factor that elevates the tHcy concentration (47,57,63), as it may inhibit the conversion of homocysteine to cysteine by acting as a vitamin B6 antagonist (63). Additionally, recent evidence became available that chlorogenic acid, a polyphenol that is present in coffee in the same amount as caffeine, may also partly be responsible for the increase in the tHcy concentration (64). When polyphenols are metabolized, methyl-groups from methionine are necessary, which results in a higher production of homocysteine (64). Both caffeine and chlorogenic acid are also present in tea, although in smaller doses, which explains the absence of an association with tea consumption.

In our population, smoking was positively associated with the tHcy concentration, which was most pronounced in women (chapter 6). Others found similar results (47,48,53,65,66). The fact that the smoking effect remained after correction for coffee consumption and folate intake (chapter 6)(48,67) excludes important confounding. Smokers generally consume a less healthy diet. Indeed, in one study the effect of smoking disappeared after correction for plasma folate (50). Correction for plasma folate concentration in place of folate intake in our study, slightly attenuated the relation between smoking and the tHcy concentration (data not shown). The

exact mechanism behind the increase in the tHcy concentration is unidentified, yet smoking may induce local effects in cells exposed to cigarette smoke (68), or may influence the tHcy concentration by changing the plasma thiol redox status (69-71), or may inhibit enzymes such as methionine synthase (72).

Alcohol consumption is probably associated with the tHcy concentration in a J-shaped fashion (53,73). For moderate alcohol consumption, we found a lower tHcy concentration in drinkers compared with non-drinkers. This inverse relation was only observed in men, probably due to the small alcohol intake range in women (chapter 6). Our findings agree with other observational studies in men (74) and men and women combined (75,76). However, two American studies found weak positive associations (47,66). As extremely high alcohol consumption is associated with elevations in the tHcy concentration (77,78), alcoholics might have drawn the positive association between alcohol and the tHcy concentration (47,66). Since we accounted for the most important lifestyle confounders (folate intake, smoking, coffee drinking), and we observed similar results after exclusion of ex-drinkers, our results suggest that moderate alcohol consumption is associated with a beneficial lower tHcy concentration compared with non-drinking. J-shaped relations between alcohol consumption and biological CHD risk factors (like HDL cholesterol) have been reported as well (79).

Considering the type of alcoholic beverage, we found that beer was probably responsible for the inverse association between alcohol consumption and the tHcy concentration, because we saw no association with wine and spirits. However, the intake range of wine and spirits was very small compared with the range of beer consumption (chapter 7: average intake in the highest tertile <0.9 glasses/d for wine and spirits, vs. 3.7 glasses/d for beer). Thus, the positive relation between beer and the tHcy concentration might also have been an ethanol effect (chapter 7). In two other population-based studies, beer consumption was associated either with a decrease (76) or no effect on the tHcy concentration (47). The results from two intervention trials with beer, wine and spirits were inconsistent: one 3-week randomized cross-over trial showed no association with the tHcy concentration after intervention with 4 glasses of beer/d, compared with an elevation of the tHcy concentration with 4 glasses/d of wine or spirits (80). Another trial, with a marginal 6-week non-randomized design in which participants could drink the alcoholic beverage of their own preference (81), showed elevations in the tHcy concentration for all three alcoholic beverage groups after daily consumption of 3 glasses/d. Hence, the results for types of alcoholic beverages are not clear. Intervention studies with lower doses of alcoholic beverages or with ethanol-water solutions may provide more insight in this issue.

Our finding with regard to a weak positive association between physical activity and the tHcy concentration (though only in women in multivariate models, chapter 6) is not in agreement with weak inverse (48,65), or no association in other observational studies (50,53). One

intervention study showed that acute exercise does not affect the tHcy concentration (82). As an active lifestyle in general is associated with a more healthy lifestyle, and a more healthy lifestyle with a lower tHcy concentration, our result is a likely chance finding, as residual confounding of e.g. smoking is expected to result in an inverse association.

Lifestyle-diet and gene-diet interactions

In chapter 4 the relation between folate intake and the tHcy concentration in males differed by the level of alcohol consumption: the slope of the relation was stronger in non-drinkers compared with drinkers. In other words, at a low folate intake level non-drinkers had a higher tHcy concentration than drinkers, whereas at a high folate intake no difference in the tHcy concentration was present between drinkers and non-drinkers (chapter 4). The observation that alcohol drinking might protect against a high tHcy level in subjects with a low folate intake was recently also described in the elderly (66-94 years) (53). These researchers observed the interaction in men and women combined. Nevertheless, the effect might be due to males, as in general the variation in alcohol consumption in men is larger than in women.

In chapter 4 we also observed effect modification of smoking on the relation between folate intake and the tHcy concentration, in men. The relation between folate intake and the tHcy concentration was much stronger in smokers: at a low folate intake smokers had a much higher tHcy level compared with non-smokers with a low folate intake. However, at a high folate intake, smokers had a comparably low tHcy concentration as non-smokers. To our knowledge, no such interaction has been reported until now.

The first gene-diet interaction with an effect on the tHcy concentration was reported by Jacques *et al* (83). At present, numerous studies have confirmed that the homozygous mutant form of the MTHFR 677 C>T polymorphism, is associated with a higher tHcy concentration especially in subjects with a sub-optimal plasma folate status (50,83-100) (chapter 5), or a sub-optimal folate intake (100) (chapter 5). However, to the best of our information the effect of the 677 C>T mutation on the relation folate intake $\rightarrow$ plasma folate, has not been shown before. This relation was positive for all genotypes, but the steepest slope was observed for CT subjects. At a low folate intake CT subjects had a low plasma folate concentration, comparable to that of TT subjects. At a high folate intake, however, their plasma folate was high, resembling that of CC subjects. In contrast, at a high folate intake TT subjects still had a lower plasma folate level than CT and CC subjects. This implies that subjects with two TT alleles need much more dietary folate to optimize their plasma folate status. Apparently, from the relation between plasma folate and the tHcy concentration, once they have achieved a high plasma folate level, the tHcy concentration of TT subjects is equally low as that in CT and CC subjects.

The finding that high folate intakes counteract the disadvantage of having one or two T alleles is explained by recent findings of Guenther *et al* (101). In a system with bacteria they

showed that a mutation homologous to the human MTHFR 677 C>T mutation was associated with an enhanced dissociation of flavin adenine dinucleotide (FAD, i.e. cofactor form of vitamin B2). An optimal folate supply prevented the loss of FAD binding and suppresses the inactivation of the enzyme (101).

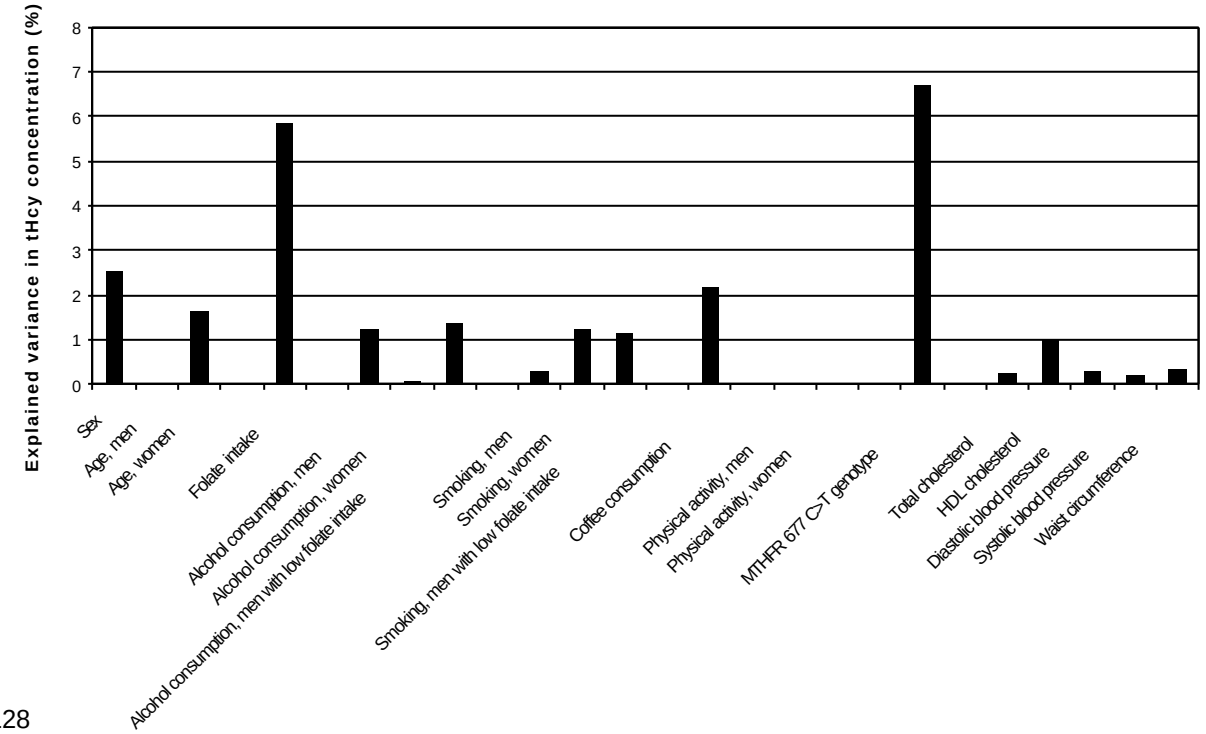
Biological CHD risk factors

In general, and also in our study (chapter 8), the associations of total cholesterol, HDL cholesterol, systolic and diastolic blood pressure and measures of body fat and the tHcy concentration are not very strong (65,102). Nevertheless, as they are associated with the tHcy concentration in the direction of an increased risk of CHD, adjustments for these factors while studying the relation between the tHcy concentration and the risk of CHD, is necessary.

Conclusions for determinants of homocysteine

Figure 1 illustrates the (univariately) explained variance in the tHcy concentration by the determinants investigated in this thesis. It is shown that folate intake is by far the most important modifiable determinant of the tHcy concentration, explaining almost 6% of the variation in the tHcy concentration. Causality can not be inferred from data of a cross-sectional study, but intervention studies (103-105) have shown that increasing the folate intake will lower tHcy concentrations.

Figure 1 Explained variance in tHcy levels (%) by various determinants investigated in this thesis (data of the cross-sectional study)



Coffee consumption is another relatively important determinant of tHcy concentration in men and women of which the causality is proven in intervention trials (61-63). Concerning the other modifiable factors, the relations of alcohol consumption (80,81) and physical activity (82) with the tHcy concentration are also investigated in intervention trials. Yet the results of these trials do not agree with findings from observational studies. This questions whether the interventions were comparable to real life situations. With regard to smoking, whether the positive relation with the tHcy concentration is a real effect, or originates from residual confounding by other lifestyle habits, remains to be elucidated. In short, more experimental information on lifestyle factors in relation to the tHcy concentration is needed.

A non-modifiable important tHcy-determinant is the 677 C>T variant of the MTHFR gene. Subjects with the TT genotype require more folate for an optimal the tHcy concentration and plasma folate status than CT and CC subjects. This should be taken into account when TT subjects are identified and treated for high tHcy levels.

Discussion of main findings: plasma homocysteine, plasma B-vitamins, and the risk of CHD

Our main findings of the prospective study described in chapter 9 are summarized in table 4. We did not find a significant association between the tHcy concentration and the risk of CHD mortality. This finding agrees with findings of 8 other prospective studies, which investigated CHD (fatal and non-fatal) as one of the endpoints of interest (74,106-112). On the other hand it disagrees with the results of 9 other prospective studies in which the tHcy concentration was found to be statistically significantly positively associated with the risk of CHD (113-121). Explanations for the lack of a significant association between the tHcy concentration and CHD will be extensively reviewed in the following section: the controversy on homocysteine and the risk of CHD.

Table 4 Relative risks[a] (95% confidence interval) of CHD mortality by plasma tHcy and B-vitamin concentrations

Exposure	Men: highest vs. lowest tertile	Women: highest vs. lowest tertile	Ch
tHcy	1.14 (0.50-2.61)	2.04 (0.48-8.62)	9
Folate	2.00 (0.82-4.87)	0.22 (0.06-0.87)	9
Vitamin B6	1.27 (0.52-3.11)	0.77 (0.22-2.63)	9
Vitamin B12	0.95 (0.40-2.29)	0.99 (0.26-3.74)	9

Explanation of symbols and abbreviations: a=adjusted for age, study center, HDL and total cholesterol level, hypertension, creatinine and smoking. Ch=chapter in this thesis.

Our finding that higher plasma folate levels are associated with a lower risk of CHD in women, agrees with other prospective studies (122-124). However, not all studies found statistically significant inverse associations (108,125). One study found a stronger inverse

relation between plasma folate and fatal CHD in women compared with men (122). In our study the inverse relation between plasma folaat and CHD was not simply less strong in men, in fact, it tended to be positive. Likely, this is a chance finding as the male cases in the highest tertile of plasma folate were on average healthy cases, e.g. they smoked less, had a lower blood pressure and a higher HDL cholesterol level (see chapter 9).

The tendency that high plasma folate levels in men are associated with a smaller (or no) risk reduction in CHD mortality could also derive from the fact that women obtain their folate from fruits and vegetables, and men obtain their folate from less healthy sources like meat. For example, the Spearman correlation between the plasma folate concentration and the intake of fibers (a proxy for fruit and vegetable intake) was stronger in women compared with men in our prospective study (women $r=0.10$, $P<0.05$ vs. men $r=0.02$, $P=0.7$). In men the folate concentration was more strongly associated with the intake of meat (women $r=0.03$, $P=0.5$ vs. men $r=0.11$, $P<0.05$). Meat consumption is associated with the simultaneous intake of other dietary compounds of animal origin (like saturated fatty acids) that are positively associated with the risk of CHD.

Evidence that plasma PLP, the biologically active form of vitamin B6 and vitamin B12 concentrations are inversely associated with the risk of CHD, was not provided by our study. Although some case-control and cross-sectional studies have shown inverse relations between PLP and the risk of CHD (42,126,127) the evidence from prospective studies is inconsistent (108,125). Vitamin B12 is probably not inversely associated with the risk of CHD (108).

The controversy on homocysteine and the risk of CHD

Inborn errors

The first and strongest evidence for elevated tHcy levels as a causal risk factor for atherothrombotic disease came from patients with inborn errors of homocysteine metabolism. When patients with a genetically determined deficiency of cystathionine β-synthase (CBS) are untreated, about 50% will have a vascular event before the age of 30 (128). Patients with other inherited defects of homocysteine metabolism, like MTHFR deficiency (129) and defects in cobalamin (vitamin B12) metabolism (130), also suffer from vascular diseases at a very young age. The common denominator in these different metabolic defects is an extremely high tHcy concentration (i.e. >100 µmol/L). Treating patients with inherited CBS deficiency with tHcy-lowering nutrients (e.g. folic acid, vitamin B12, B6 and betaine) prevents vascular events at young age (131). Despite treatment, the tHcy levels of these patients are well above the normal range (i.e. >30 µmol/L)(131). This could mean that the relative risk of these patients to develop premature CHD (<60 years) is still higher compared with persons with normal tHcy levels.

Retrospective, cross-sectional and prospective studies

Earlier retrospective and cross-sectional studies have consistently showed a stronger relation with the tHcy concentration than the more recent prospective studies (132-135). The meta-analysis of Boushey *et al* (132) in 1995 summarized 11 retrospective and cross-sectional studies, which all showed a significant increased risk of CHD for each 5 µmol/L increase in the tHcy concentration (odds ratios all ≥1.5). Of the 2 prospective studies available at that time, one found a positive association (116) and one did not find an association (106). In 1998, this meta-analysis was updated by Refsum *et al* (102). Of the additional 16 retrospective and cross-sectional studies, which evaluated CHD as one of the endpoints of interest, only 3 did not find a positive association between the tHcy concentration and CHD risk. One additional population-based prospective study (117) showed a significant positive relation between the tHcy concentration and the risk of CHD.

In 1998 another meta-analysis was performed that calculated the relative risk (RR) for prospective and retrospective studies separately (134). The summary RR of CHD from all available retrospective studies with population-based controls was 1.6 (95% confidence interval (CI)=1.4-1.7). For prospective studies this RR was lower: 1.3 (95% CI=1.1-1.5). In 2000 the totality of evidence was evaluated qualitatively (133) and the results from prospective studies were in general weaker (or absent) in comparison with those of retrospective and cross-sectional studies. Nevertheless, the most recent meta-analysis based only on prospective studies with subjects not selected for their increased risk of CHD (135), calculated that each 5 µmol/L increase in the tHcy concentration was associated with a 20% increase in risk of CHD (odds ratio: 1.2, 95% CI=1.1-1.3). Table 5 gives an overview of all the prospective studies included in this meta-analysis, and is updated with the most recent studies.

There are several reasons for the different results between cross-sectional and retrospective studies on the one hand, and prospective studies on the other hand. These will be evaluated in the following paragraphs. Furthermore, explanations for different results between prospective studies will also be discussed.

First, the issue of the chronological sequence between collecting information on study subjects (including blood drawing) and the occurrence of the disease. In retrospective studies data collection takes place after the CHD event. This means that the event might have distorted recall of certain lifestyle (such as smoking) and dietary habits. Furthermore, it implicates that medical treatment, like lipid-lowering drugs, anti-hypertensive therapy, or smoking cessation, may have altered the levels of traditional CHD risk factors. Moreover, the disease could have influenced blood levels of tHcy, but also of risk factors like blood pressure and cholesterol level (107). In cross-sectional studies, tHcy levels and extent of the disease are assessed at the same moment in time. As cases are typically persons with early signs of vascular disease (e.g.

angiographically confirmed stenosis), the effect of the disease on CHD risk factors like blood pressure and cholesterol level cannot be excluded (133). Prospective studies have the major advantage that blood is collected before the event, thus no influence of the disease on lifestyle and dietary habits, and blood parameters is expected, if subjects with vascular diseases at baseline are excluded.

Box 2 The influence of vascular disease on tHcy concentrations

Several studies have measured tHcy levels on the day of a CHD event and compared it with the levels measured on days after the event (up to 180 days after the event) (136-138). Typically they observed lower tHcy concentrations on the day of the event compared with the concentrations on the days after the event. As no study measured tHcy levels just before the event, it can not be excluded that tHcy concentrations were lower during the event and over time return to the normal level from before the event. In addition, a rise in tHcy concentration may be due to the fact that a patient is going from an active life to bedridden; i.e., the tHcy concentration in blood collected in supine position is higher than that collected sitting (maximally 30% higher) (7).

A metabolic reason for an increase in the tHcy concentration after the event is provided by Dudman (139). He suggests that the increase in the tHcy concentration is the result of tissue damage and repair. Tissue repair involves synthesis of DNA, RNA and proteins that require methyl-groups. Most methyl-groups originate from methionine and when methionine donates its methyl-group, homocysteine is generated (see figure 1, General Introduction). Dudman postulates that a higher tHcy concentration attracts leukocytes to the vascular endothelium, where they play a role in tissue repair and remodeling (139). However, to our knowledge no studies have been done to test whether induced vascular damage increases tHcy concentrations.

Another mechanism that could explain a higher tHcy level in subjects with CHD is proposed by Brattstrom and Wilcken (140). They reviewed currently available evidence and propose that atherosclerosis caused by traditional cardiovascular risk factors (such as high blood pressure and smoking) impairs the renal function. As the kidneys are quantitatively important for the catabolism of homocysteine, this might lead to higher tHcy concentrations.

The fact that the risk estimates of retrospective and cross-sectional studies are generally higher compared with prospective studies, could on the one hand be explained by higher tHcy levels after a vascular event (136) (see box 2 for further discussion of this issue). On the other hand, if the disease and/or medical treatment modify the levels of CHD risk factors, then retrospective and cross-sectional studies might also show higher risk estimates. Because adequate control of the confounding effect of these risk factors on the association between the tHcy concentration and the risk of CHD is not possible due to the masking of “true” levels of these risk factors (107).

Second the issue of the simultaneous presence of higher tHcy concentrations and CHD irrespective of whether these higher levels are the cause or the consequence of CHD. Due to this issue one can assume that prospective studies that included subjects with pre-existing CHD, in theory, should show a more consistent statistically significant positive association between the tHcy concentration and the risk of CHD. Of the studies that are summarized in table 5, 3 studies offer support for this assumption. Stehouwer *et al* (109) showed that the tHcy concentration was more strongly associated with the recurrence of an event rather than a first ever myocardial infarction. Vollset *et al* (120) observed that after stratification for high and low baseline risk (high risk indicated a history of myocardial infarction, stroke, angina pectoris,

diabetes, or hypertension), only high-risk persons showed a significant association between the tHcy concentration and cardiovascular mortality. Furthermore, besides the findings presented in table 5, Knekt *et al* (112) reported separately on men with prevalent CHD at baseline. In these men, a higher tHcy concentration was significantly associated with the risk of CHD mortality and morbidity. In our study the RR of CHD mortality in men and women with a history of CHD or cerebrovascular accidents was larger than the estimate for men and women free of baseline cardiovascular diseases (1.58, 95% CI=0.81-3.09 vs. 1.03, 95% CI=0.83-1.29, for each 5 µmol/L increase in the tHcy concentration). However, the number of subjects with baseline cardiovascular diseases was too small to show a significant RR (chapter 9). Finally, in elderly the chance of silent, pre-clinical, CHD is larger. Of the five prospective studies with elderly populations (average age >60 years) (109,114,118,120,121), only one (109) did not show a significant association between the tHcy concentration and the risk of CHD.

Prospective studies performed in high-risk populations consistently show that the tHcy concentration is a strong predictor of cardiovascular mortality and morbidity (including CHD) in subjects with CHD (141), diabetes (142-144), renal insufficiency (145,146), peripheral artery disease (147), and systemic lupus erythematosus (148).

The third issue is the aspect of the duration of follow-up. If the tHcy concentration is a risk factor in high-risk subjects, follow-up studies may show a stronger relation between the tHcy concentration and CHD during the first few years of follow-up, because subject with pre-clinical disease will decease earlier than those without pre-clinical disease. This is confirmed by data of the Physicians' Health Study. After 5 years of follow-up a significant increased risk of CHD was found in men with elevated tHcy levels (116), however, extending the follow-up to 7.5 years yielded a non-significant RR (125). Furthermore, in the same study population no significant association was observed between the tHcy concentration and the risk of angina pectoris after 9 years of follow-up (149). Two other studies also showed a stronger relation between the tHcy concentration and CHD mortality (109) and total cardiovascular disease mortality (121) during the first few years of follow-up. Finally, the studies in table 5 with a short follow-up period (<5 years) showed in general more often a significant association between the tHcy concentration and the risk of CHD. These results indicate that the tHcy concentration might be a short–term risk factor for CHD.

An additional feature of prospective studies with a long follow-up is that a reduction in the risk estimate may also occur as a result of changes in diet, lifestyle, or medical treatment during follow-up. These factors may alter the tHcy concentration in such a manner that the baseline tHcy concentration, is no longer representative for the concentration at the time of the event. In addition, due to a combined effect of measurement errors and intra-individual variation, the "usual" level of tHcy that is related to the risk of CHD might be difficult to approach with a single tHcy measurement. The result of this so-called regression dilution bias, is an attenuated

association between the tHcy concentration and the risk of CHD. This bias can be estimated and corrected for by taking more blood samples over the period of follow-up and using the data of replicate tHcy measurements (150,151).

In conclusion, the differences in strength of the association between prospective studies and retrospective and cross-sectional studies can be ascribed to the fact that in prospective studies data is collected before the event. Prospective studies with subjects not selected for their risk of CHD show in general a weak association between the tHcy concentration and the risk of CHD. Yet, ordering prospective studies into studies with and without subjects with pre-existing CHD, with younger and older subjects, and with a long and a short follow-up, shows that associations are more consistently found in studies including subjects with CHD, with elderly and with a short follow-up. Prospective studies in high-risk populations consistently show a strong relation between the tHcy concentration and CHD (RRs >2). These results could either mean that the tHcy concentration is a short-term risk factor in subjects with a high risk of CHD, as suggested by some researchers (107,152), or it could mean that elevations in the tHcy concentration are merely a marker of the degree of the underlying vascular disease.

Other studies that give insight into whether the tHcy concentration is a marker of disease, or causally related to the development of CHD are evaluated in the next paragraphs.

Homocysteine and thrombosis

As will be mentioned more extensively in the section on mechanisms, elevated tHcy level might interfere with normal coagulation and fibrinolysis. There is considerable epidemiological evidence that the tHcy concentration is a risk factor for venous thrombosis (153,154). The 3 prospective studies on the relation between the tHcy concentration and venous thrombosis all show a significant positive association in subjects healthy at baseline (155), subjects with systemic lupus erythematosus (148) and in subjects with a history of venous thrombosis (156). In addition, thrombotic disease is responsible for 50% of the vascular events in patients CBS deficiency (128). If the tHcy concentration is more a thrombogenic factor than an atherogenic factor, part of the weak association between the tHcy concentration and the risk of CHD could theoretically be due to the fact that CHD comprises heart diseases with both a thrombogenic and atherogenic origin.

A primary thrombogenic effect of the tHcy concentration might explain why it is consistently associated with an increased risk of CHD in high-risk subjects. If these subjects already have a certain degree of atherosclerosis, tHcy-induced thrombosis might be the crucial factor causing vascular occlusion.

Table 5 Prospective studies on plasma tHcy concentrations and risk of (non-) fatal CHD in populations not selected on the basis of risk of CHD[a], adapted from (135)

Reference	Study	Follow up years	Age	Sex	Outcome	At baseline exclusion of:	RR for each 5-µmol/L increase in tHcy[a]
Stampfer *et al*, 1992 (116)	Physician's Health Study, USA	5	40-84	M	fatal and non-fatal MI and CHD	past history of MI, stroke, or TIA	1.29 (1.01-1.64)
Alfthan *et al*, 1994 (106),	North Karelia Project, Finland	9	40-64	M, F	fatal and non-fatal MI	past history of CVD	1.03 (0.66-1.53)
Arnesen *et al*, 1995 (117)	Tromsø Health Study, Norway	4	34-61	M, F	fatal and non-fatal CHD	past history of MI	1.41 (1.06-1.88)
Evans *et al*, 1997 (107)	MRFIT, USA[b]	11+	35-57	M	non-fatal MI, fatal CHD	past history of morbidity (not explained)	0.98 (0.83-1.15)
A'Brook *et al*, 1998 (113)	Scotland	7.6	35-64	M, F	CHD, unclear whether fatal or non-fatal	?	1.50 (1.28-1.78)
Ubbink *et al*, 1998 (74)	Caerphilly, UK	5	50-64	M	fatal and non-fatal IHD	no, adjustments for prevalent CHD	1.22 (0.88-1.64)
Folsom *et al*, 1998 (108)	ARIC, USA	3.3	45-64	M, F	fatal and non-fatal CHD	past history of CHD, stroke, or TIA	1.15 (0.68-1.92)
Wald *et al*, 1998 (115)	BUPA, UK	8.7	35-64	M	fatal IHD	past history of CHD	1.41 (1.20-1.65)
Stehouwer *et al*, 1998 (109)	Zutphen, the Netherlands	10	64-84	M	fatal and non-fatal CHD	no, adjustments for prevalent CHD	1.05 (0.97-1.15)
Bostom *et al*, 1999 (114)	Framingham, USA	10	59-91	M, F	fatal CHD	?	1.42 (1.13-1.77)
Bots *et al*, 1999 (118)	Rotterdam, the Netherlands	2.7	>55	M, F	fatal and non-fatal MI	no, adjustments for prevalent CHD	1.28 (1.05-1.76)
Ridker *et al*, 1999 (119)	Women's Health Study, USA	3	mean 59	F	fatal and non-fatal CHD	past history of CVD	1.74 (1.13-2.64)
Whincup *et al*, 1999 (110)	BRHS, UK	12.8	40-59	M	fatal and non-fatal MI	no, adjustments for prevalent CHD	1.13 (0.99-1.29)
Kark *et al*, 1999 (121)	Jerusalem Study, Israel	9-11	>50	M, F	fatal CHD	no, association remained after exclusion of history of CVD	1.34 (1.05-1.62)
Voutilainen *et al*, 2000 (111)	KIHDRF Study, Finland	8,9	42-60	M	fatal and non-fatal CHD	history of CHD	0.88 (0.44-1.76) in highest tHcy quartile vs. lowest quartiles
Vollset *et al*, 2001 (120)	Hordaland elderly, Norway	4.1	65-67	M,F	fatal CHD	no, adjustments for prevalent CHD	1.46 (1.14-1.87)
Knekt *et al*, 2001 (112)	MCHES, Finland	13	45-64	M	fatal and non-fatal CHD	history of CHD	0.90 (0.51-1.60) in highest tHcy quintile vs. lowest quintile
de Bree *et al* 2001 (chapter 9)	MPCDRF, the Netherlands	10.3	20-59	M,F	fatal CHD	history of CVD	1.03 (0.83-1.29)

Explanation of symbols and abbreviations: a=unless indicated otherwise; b=these men were selected for a baseline moderate risk of CHD, based on a combination of their total cholesterol level, diastolic blood pressure and smoking habits; TIA=Transient Ischemic Attack, MI=Myocardial Infarction, CVD=Cardiovascular Diseases, IHD=Ischemic Heart Disease; ?=unclear if subjects with cardiovascular diseases at baseline were excluded.

MTHFR 677C>T genotype and CHD

A person's genotype of the 677 C>T variant of the MTHFR gene is present from birth onwards and will not change over the years. The TT variant of this genotype leads to an approximately 25% higher tHcy concentration compared with CC subjects (157). Nevertheless, this genotype has not consistently been associated with CHD (157,158). These inconsistencies might be attributable to a power problem (159). The average difference in tHcy concentration between TT and CC subjects ≈2.6 µmol/L (157). According to a pooled RR estimate based on prospective studies, this difference in tHcy concentration might produce a RR of 1.10-1.15 (135). The calculated point estimate of a meta-analysis (1.12, 95% CI=0.92-1.37) performed by Brattstrom *et al* (157), though not statistically significant, is in line with an effect of this magnitude. To identify a statistically significant RR of this size with a statistical power of 80%, one needs between 7800-16,300 cases and an equal number of controls (135). Currently the largest meta-analysis with ~8000 cases and ~8000 controls is being performed and the outcome will give a more definite answer to the question whether this genotype is related to an increased risk of CHD (160).

Mechanism by which homocysteine increases the risk of CHD

Investigations of patients with homocystinuria, animals and *in vitro* studies identified several means by which the tHcy concentration may cause atherosclerosis and thrombosis. These include endothelial injury, platelet activation, smooth muscle cell proliferation, oxidation of blood lipoproteins like LDL-cholesterol, and endothelial leukocyte interaction (161). The diversity in effects may reflect a true variety, but may also be due to the difficulty to mimic atherosclerotic processes in short term *in vitro* studies, or to account for *in vivo* interrelations between aminothiols, like homocysteine and cysteine (102). In addition, many of the above mechanisms were shown in *in vitro* studies with non-physiologically high tHcy concentrations. Numerous experiments were done with tHcy concentrations of 1000 to 10,000 µmol/L, in comparison, untreated patients with inborn errors can have tHcy levels up to 400 µmol/L. In addition, a lot of studies used free reduced homocysteine. As only 1% of the tHcy concentration in plasma is free reduced homocysteine, the experimental concentrations sometimes approached levels that are 50,000 times higher than the level that can be found in humans (161). Finally, it can be difficult to disentangle effects of homocysteine from other thiols like cysteine (102,161).

Despite these critical remarks, to date one of the most plausible mechanisms by which homocysteine may cause atherosclerosis and thrombosis may be by endothelial dysfunction (161-163). Endothelial cells play a vital role in regulating and maintaining vascular health. In addition, endothelial cells are essential to hemostatic processes of cell adhesion and migration, coagulation and fibrinolysis (162). A key regulatory system of endothelial cells involves nitric

oxide synthase, which synthesizes nitric oxide (NO) (163). Endothelial derived NO regulates vessel tone, inhibits platelet activation, adhesion and aggregation, limits smooth muscle cell proliferation and modulates endothelial-leukocyte interaction (161). At normal tHcy concentrations homocysteine reacts with NO to form S-nitroso-homocysteine, which has some properties of NO; it inhibits platelet aggregation, it is a vasodilator and it prevents the formation of reactive oxygen species (ROS). However, when endothelial cells are continuously exposed to higher tHcy concentrations the result is a downwards spiral in which endothelial cells are no longer able to reduce the toxicity of homocysteine (164,165). Besides a possible direct effect of homocysteine on endothelial cells, it may also reduce NO activity by the formation of ROS (166,167) and by inhibition of glutathione peroxidase (168,169), which is an important enzyme protecting the endothelial cell against oxidative stress.

As NO regulates the vessel tone, several studies have measured the endothelium-dependent flow-mediated vasodilatation in response to homocysteine. Impaired endothelium-dependent, flow mediated vasodilatation has been documented in subjects with high tHcy concentrations (170,171), in healthy subjects after a methionine-load induced high tHcy level (172,173), and in healthy subjects after a physiologically higher tHcy concentration, generated by a protein rich meal (174).

Intervention trials

Because of the tHcy-lowering effect of folic acid, several studies investigated the effect of folic acid supplementation on intermediate endpoints of vascular damage. Endothelial dysfunction is such an intermediate endpoint. Brown and Hu recently reviewed trials that considered the effect of folic acid supplementation on endothelial function (162). The general picture that emerges from these studies is that folic acid (5 to 10 mg/d) improves or restores endothelium-dependent vasodilatation and may decrease the chance of thrombosis by reducing levels of coagulation factors in healthy subjects and in patients with high tHcy levels (162). The observed benefit is probably largely explained by the lowering of tHcy concentrations. However, in one study folic acid infusion improved endothelial function, without an effect on the tHcy concentration (175). In the latter study folic acid infusion reduced oxidative stress (175). Moreover, another study showed that folic acid might improve NO production through stimulation of NO-synthase (176). Thus, folic acid may have a beneficial effect on endothelial function independent of the tHcy concentration.

In light of these results, it is interesting to mention that Chambers *et al* (177) suggest that the lack of a strong association between lower tHcy levels, in response to folic acid therapy, and improved endothelial function is due to the measurement of *total* homocysteine as the only index of the homocysteine status. Box 3 describes this more extensively.

Currently there are 3 non-controlled trials of which 2 show that supplementation with folic acid (5000 µg) and vitamin B6 (250 mg) reduces the risk of cardiovascular events (coronary, peripheral and cerebral) in patients with high tHcy levels and existing cardiovascular diseases, to the level of patients with existing cardiovascular diseases, but with normal tHcy levels (180,181). The other trial investigated the effect of folic acid (2500 µg), vitamin B6 (25 mg) and vitamin B12 (250 µg) on the regression of carotid plagues. Vitamin supplementation resulted in a decreased rate of progression of the plague growth in 101 patients with vascular disease with normal and elevated tHcy levels (182).

Box 3 Non-protein bound and reduced free homocysteine as an index of the biological activity of homocysteine

Chambers *et al* (177) showed in a randomized placebo controlled intervention study in 89 male CHD patients, that endothelium-dependent flow mediated dilatation improved after 8 weeks of supplementation with folic acid and vitamin B12. This improvement correlated significantly with a reduction in non-protein-bound homocysteine (~30% of the tHcy concentration), but was independent of changes in protein-bound homocysteine and plasma folate and vitamin B12. Furthermore, in another study of their group with 14 healthy volunteers (10 men, 4 women) (178), they found that the reduction in flow-mediated dilatation maximally correlated with concentrations of free reduced homocysteine (~1% of the tHcy concentration), but not with the other forms of homocysteine.

The correlation between the tHcy concentration and reduced free homocysteine is small (0.4, $p<0.05$) (69), which excludes that the tHcy concentration can be used as a proxy for the free reduced homocysteine concentration. Measuring free reduced homocysteine is a complicated procedure, requiring immediate blood sampling processing due to rapid changes in redox status of free aminothiols *ex vivo* and stored plasma samples cannot be used (179). It therefore seems unlikely that the hypothesis that free reduced homocysteine is responsible for atherothombosis (178) can be confirmed in large scale prospective epidemiological studies.

The only available randomized, placebo controlled trial with intermediate endpoints was performed with 158 healthy siblings (without prevalent venous thrombosis, clinical arterial disease and renal impairment) of patients with sub-clinical cardiovascular diseases. They received a daily supplement either with 5000 µg folate and 250 mg vitamin B6 (n=78), or placebo tablets (n=80), for a period of 2 years (183). In the treatment group the tHcy concentration decreased as expected. Treatment was associated with fewer abnormal exercise electrocardiography tests, which is a measure (though with internal validity shortcomings (184)), of coronary atherosclerosis. An effect of treatment on other surrogate outcome measures (ankle-brachial pressure index and duplex-scanning of the carotis and peripheral arteries) was not observed (183).

The results of the above mentioned intervention trials with intermediate endpoints are in favor of the hypothesis that lower tHcy concentrations are causally associated with a decreased risk of vascular disease in patients with cardiovascular diseases (180-182) and in healthy subjects (183). Yet, in all intervention trials folic acid was used. As this vitamin may have a favorable effect on e.g. endothelial function (175,176) independently of the tHcy concentration,

it is not clear whether the observed effect is due to a direct folic acid effect or to a lower tHcy concentration.

Ongoing intervention trials will answer the question whether a lower tHcy concentration through vitamin supplementation (folic acid, vitamin B6 and B12) has an effect on “hard” end points, like CHD mortality (185,186). The results of these trials will become available within 2-4 years. If these trials show a reduction in CHD (or other vascular endpoints), this does not answer the question whether the tHcy concentration is a causal risk factor in healthy subjects, as all trials included high-risk populations to increase the statistical power. Note, that it is questionable whether the trials performed in the USA are able to show a reduction in CHD, as they will likely suffer from a lack of power due to the mandatory folic acid fortification since 1998 (187).

A question that also will not be answered by the currently ongoing trials is whether a lower tHcy concentration or a higher B-vitamin intake (or both) is the cause of less CHD. This answer can only be provided in trials using other tHcy-lowering compounds, like betaine (188).

Conclusion about the causal relation between homocysteine and CHD

Table 6 weighs the available evidence to the extent that it offers support for the tHcy concentration as a causal risk factor for CHD. It is beyond reasonable doubt that the extremely high tHcy levels of patients with inborn errors cause CHD. However, what is the relevance of this causal relation for the relation between moderately elevated tHcy levels and CHD? Moderately elevated tHcy levels are associated with CHD in high-risk subjects, like with diabetes, or with a history of CHD. In these patients an increased tHcy concentration might provoke the event, resulting in a short-term association with the risk of CHD. Nevertheless, the tHcy concentration might just as well be a marker of the degree of vascular disease.

Current epidemiological evidence does not provide strong evidence that elevations in the tHcy concentration are harmful in healthy subjects. Yet, lowering tHcy levels through administration of folic acid and vitamin B6 favorably influenced the progress of atherosclerotic disease in healthy subjects measured with an exercise electrocardiography tests (183). The results of another primary intervention trial with healthy subjects and well-validated intermediate endpoints (carotid wall thickness and stiffness) will be available in 2004 (189).

Table 6 Evidence for homocysteine being causally involved in the etiology of CHD

Type of evidence	Extent to which it offers support	Comment
Inborn errors	++++	Untreated, these patients suffer or even die of vascular disease, treatment prevents or delays vascular events
Retrospective and cross-sectional studies	+	Due to the fact that blood sampling occurs after the event, the effect of CHD on tHcy levels can not be excluded
Prospective studies with healthy subjects	+	Evidence is stronger in older subjects and in studies with a short follow-up period
Prospective studies with high-risk subjects	++	In these types of studies it can not be excluded that the increased levels of tHcy are a marker of the degree of vascular disease
Prospective studies with venous thrombosis	+	These studies indicate that the tHcy concentration may predominantly be a thrombogenic factor, yet the number of prospective studies is small
MTHFR 677 C>T genotype	+	Lack of evidence that the TT genotype is associated with CHD may be a power problem
Mechanism of action	++	Especially the relation with endothelial function seems plausible
Intervention trials with intermediate endpoints	+++	Beneficial effect could also be the result of folic acid

Explanation of symbols: + indicates minor support, ++++ indicates strong support.

Thus, anticipating on the ‘Directions for future research’, the only study that can answer the question whether the tHcy concentration is a causal risk factor for CHD in healthy subjects, is an intervention trial with healthy subjects, hard end-points and tHcy-lowering nutrients other than folic acid. This trial should be carried out in apparently healthy subjects (e.g. free of cardiovascular diseases and diabetes) with elevated tHcy concentrations to avoid a power problem. Betaine could be used as a tHcy-lowering nutrient (188). It is, however, more likely that such a trial will be initiated after the results of the secondary intervention trials are available. If these trials do not show a reduced incidence of CHD in treated high-risk subjects, this may not justify an intervention study in healthy subjects.

Directions for future research

Pending the results of the intervention trials, more epidemiological (prospective) studies on the tHcy concentration and cardiovascular diseases are not desirable, as they cannot provide the ultimate answer for the causality question.

Future research should focus on experiments that elucidate which *form* of homocysteine (reduced, (non-) protein bound) might cause atherosclerosis and/or thrombosis and by which etiologic *pathway*. A promising pathway involves endothelial dysfunction. So far, endothelial function in response to high tHcy levels, or in response to folic acid administration, have only been investigated in small groups of healthy subjects. Future studies should include larger groups of healthy subjects. In addition, to disentangle whether the increase in folic acid or the reduction in the tHcy concentration are beneficial for the endothelium, it would be interesting to explore the effects of betaine, vitamin B6, B12 or B2 supplementation on endothelial function.

Irrespective of whether beneficial effects on the endothelial function are due to a lower tHcy concentration, or due to a higher folate concentration, in both cases a higher folate intake will improve endothelial function. Thus, research on the desired folate intake level to achieve beneficial endothelial responses is necessary. For example, endothelium-dependent vasodilatation and other responses associated with endothelial function (improved coagulation) could be monitored in experiments in which participants are provided with folate rich meals containing different doses of folate.

A research question that remains interesting to investigate in observational studies is to what extent the tHcy-determinants described in this thesis are important in subjects with a high-risk of vascular disease. For example, in the general population drinking 6 cups of coffee/d is associated with an average increase in the tHcy concentration of 1.3 µmol/L. It is not known how strong this relation is in subjects with cardiovascular diseases or in other high-risk subjects.

Finally, effort should be taken to standardize the measurement of the tHcy concentration. If it turns out that the tHcy concentration causes vascular diseases, it will be of utmost importance to precisely and validly measure the concentration. Otherwise, an accurate estimate of a person's risk can not be made.

Implications for prevention and treatment

Although definitive proof for a causal role of the tHcy concentration in the etiology of CHD is lacking, even a moderate effect of the tHcy concentration on the occurrence of CHD deserves attention, particularly since simple, safe, and inexpensive treatments exist that can lower the tHcy concentration. As folate is the most important modifiable factor of the tHcy concentration, it seems attractive to use folate as a means to reduce the tHcy concentration. In the USA, folic acid fortification has proven to effectively lower tHcy levels (190,191). However, folic acid fortification has the negative side effect that it may mask a vitamin B12 deficiency by correcting the hematological, but not the neurological, abnormalities of vitamin B12 deficiency. Based on this masking effect of folic acid, the Health Council of the Netherlands decided that only products specifically intended for women who want to become pregnant, may be fortified with folic acid (192). It is possible that this recommendation will be reconsidered as soon as evidence from the secondary trials described above becomes available. Note, however, that the ongoing discussion of the masking of a vitamin B12 deficiency would become irrelevant when fortification with folic acid would be accompanied by simultaneous fortification with vitamin B12.

The increased attention on the tHcy concentration has created a demand for guidelines by general practitioners and specialists, like cardiologists. As a result of this, the Netherlands Heart Foundation has published a report that contains temporary guiding principles, pending the results of intervention trials. For the Dutch situation, a “screen and treat” scenario is suggested (193). In addition to avoiding negative side effects of the “treat all” scenario by fortification, this scenario is probably more cost-effective than a treat all scenario (194).

Persons that are eligible for screening, i.e. the measurement of their tHcy concentration, are those with a high risk of CHD, e.g. patients with diagnosed cardiovascular occlusions, thrombosis, diabetes, renal insufficiency, and an unfavorable history of CHD in the family. Subjects with an elevated tHcy concentration can be treated with a daily supplement of 500 µg/d of folic acid as this was proven to be equally effective in lowering the tHcy concentration, as supplements with higher doses (195). Depending on the laboratory the definition of elevated tHcy concentrations may differ, but generally a level above >15 µmol/L (196) is considered elevated. Patients that are treated with folic acid should be followed up regularly, to monitor their vitamin B12 status and to see whether their tHcy concentration decreases. If the tHcy concentration does not decrease, a higher dose of folic acid can be chosen, or additional ways to lower the tHcy concentration may be used, like administration of vitamin B6 or betaine. The tHcy-lowering treatment should never interfere with the established treatments to prevent CHD, like cholesterol-lowering medication, anti-hypertensive treatment, and encouragement of a healthy lifestyle (193).

For subjects without a high risk of CHD, an optimal folate intake might be beneficial as well, since a low folate concentration or intake is not only inversely associated with the risk of CHD, but also with colon cancer (197,198), pregnancy complications (199) and dementia (200-202). Therefore, it seems worthwhile to initiate and maintain public health educational programs targeted at increasing the consumption of plant foods, like the currently ongoing campaigns of the Fruit and Vegetable Information Bureau, and the Netherlands Nutrition Centre. Furthermore, stimulating a healthy lifestyle with moderate coffee and alcohol consumption, and no smoking, will contribute to a lower tHcy concentration. This will lead, also independently of the tHcy concentration, to a lower incidence of CHD.

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SUMMARY

Cardiovascular diseases, and especially coronary heart disease, are the most important cause of death in industrialized countries. Major risk factors of coronary heart disease are a high total cholesterol level and/or low HDL cholesterol level, smoking and a high blood pressure. Recently increased concentrations of plasma homocysteine also have been associated with an increase risk of coronary heart disease.

Homocysteine is an amino acid that is not incorporated in proteins, nor is it present in foods in significant quantities. It is formed intracellularly when the amino acid methionine is metabolized. The intracellular concentration of homocysteine is precisely regulated and any excess is transported into plasma. Increased concentrations of homocysteine in plasma may stimulate atherosclerosis and/or thrombosis.

To date there is no definitive proof that the association between raised homocysteine concentrations and coronary heart disease is causal. However, assuming that this relation is causal, a lower homocysteine concentration will have a beneficial effect on the occurrence of coronary heart disease. Therefore, it is important to know which factors determine the homocysteine concentration.

This thesis describes cross-sectional studies that investigated to what extent dietary, lifestyle, genetic, and biological coronary heart disease risk factors determine the homocysteine concentration. Furthermore, the relation between elevated homocysteine concentrations and the risk of coronary heart disease mortality was quantified in a prospective study. The cross-sectional and the prospective study populations comprised adults (20-65 years) representative for the general Dutch population.

The potential of folic acid, the synthetic form of folate present in supplements, to lower the extremely high homocysteine concentrations of patients with inborn errors of homocysteine metabolism was recognized already several decades ago. The question remained whether higher intakes of folate (from the dietary and/or supplements) would result in lower homocysteine concentrations in the general population. The available literature on this topic at the time of 1997 is summarized in **CHAPTER 2**. In this review we described the recommended, actual and desired folate intake. The literature search was focused on European adult populations. As there was no threshold homocysteine level above which the risk of coronary heart disease increased and as the risk of coronary heart disease was assumed to decrease continuously with a decreasing homocysteine concentration, we estimated the desirable folate intake in light of an optimally low homocysteine concentration.

The most important conclusions of this review were that the mean dietary folate intakes in Europe (men: 291 µg, women: 247 µg per day) were in line with the recommendations (170-300 µg per day), but were lower than the desired dietary intake of more than 350 µg per day necessary for optimally low homocysteine levels.

If homocysteine is a causal risk factor for coronary heart disease, then public health authorities need information on the proportion of subjects that might be "at risk". This proportion can be derived from the homocysteine distribution curve when from a practical point of view a cut-off point for elevated homocysteine levels is chosen. Due to the continuous association between homocysteine and the risk of coronary heart disease every cut-off point is arbitrary. In **CHAPTER 3** we provided a homocysteine distribution curve for the Dutch population. For this purpose we measured the non-fasting homocysteine concentration in a random sample of 3025 men and women aged 20-65 years. The mean homocysteine concentration for men was 14.6 (minimum 5.9 and maximum 94.6) µmol/L and for women 13.1 (minimum 6.1 and maximum 72.2) µmol/L.

A prominent laboratory in Bergen, Norway, uses 15 µmol/L as a cut-off point to indicate elevated homocysteine concentrations. We could not simply use this value as the homocysteine concentration of one sample measured in different laboratories can vary considerably. To compare homocysteine values we performed a comparative study for which the homocysteine concentration of 301 plasma samples were measured in our laboratory (in Nijmegen) and in Bergen. The result was a systematically higher homocysteine concentration in Nijmegen of –2.4 µmol/L. Taking this systematic difference into account, the prevalence of high homocysteine levels (>17.4 µmol/L) was 14.4% in men and 8.9% in women. In comparison with data of other European studies, for which the homocysteine concentration was also measured in Bergen, the Dutch values (after correction for the systematic difference) were in general higher.

As mentioned before, information on the relation between dietary folate intake and homocysteine concentration in the general population was limited. Furthermore, information on the relation between the other B-vitamins essential in homocysteine metabolism and homocysteine levels was also insufficient. In **CHAPTER 4** we described the association between dietary intakes of folate, riboflavin (vitamin B2), vitamin B6 and B12 and the homocysteine concentration. For this study we used a sub-group (n=2435) of the above mentioned random sample. Higher intakes of all B-vitamins were associated with lower plasma homocysteine concentrations. However, after correction for confounding variables (other B-vitamins, methionine, age, smoking and alcohol consumption) only folate was inversely associated with the homocysteine concentration. An average low folate intake (men: 161, women: 160 µg per day) was associated with a high tHcy concentration, in men on average 15.4 µmol/L and in

women on average 13.7 µmol/L. An average high folate intake (men: 254, women: 262 µg per day) was associated with a low homocysteine concentration, in men on average 13.2 µmol/L and in women on average 12.4 µmol/L. In men, the difference in the mean plasma homocysteine concentration between low and high folate intakes was greater in smokers than in non-smokers (2.8 vs. 1.6 µmol/L), and greater in non-drinkers than in drinkers of more than 2 alcoholic drinks per day (3.5 vs. 1.4 µmol/L). We concluded that in this Dutch population, folate was the only B-vitamin independently inversely associated with the plasma homocysteine concentration. A higher dietary folate intake may substantially influence the plasma homocysteine concentration.

Besides B-vitamins, the activity of enzymes that are involved in homocysteine metabolism is also important for the homocysteine concentration. The enzyme 5,10-methylene-tetrahydrofolate reductase (MTHFR) produces the main circulating form of folate that is necessary for the conversion of homocysteine to methionine. A mutation in the gene encoding for MTHFR, the 677 C>T polymorphism, decreases the enzyme activity. The activity is normal in subjects with the CC genotype, somewhat reduced in CT subjects and clearly limited in TT subjects. In **CHAPTER 5** we described the consequences of this genotype for folate intake, plasma folate and homocysteine concentration. Again we cross-sectionally analyzed a sub-group (n=2051) of the random sample. The relation between folate intake and plasma folate was positive in all genotypes. The steepest relation was seen in CT subjects: at a low folate intake (mean: 155 µg per day) the plasma folate concentration of CT subjects resembled that of TT subjects (CT: 5.8, TT: 5.7 nmol/L), but at a higher folate intake (mean: 267 µg per day) the plasma folate concentration resembled that of CC subjects (CT: 8.4, CC: 8.7 nmol/L). In TT subjects, the plasma folate concentration was always lower than that of CT and CC subjects despite equally high folate intakes. The relation between the concentration of plasma folate and homocysteine was inverse in all genotypes and most pronounced in TT subjects: at a low plasma folate concentration (mean: 4.0 nmol/L) the homocysteine concentration was much higher for TT subjects than for the other genotypes (TT: 20.9, CT: 15.4, CC: 14.3 µmol/L), while at high folate concentrations (mean: 13.7 nmol/L) the difference in homocysteine concentration between the genotypes was negligible. The relation between folate intake and homocysteine was also inverse, and most pronounced in TT subjects. Our results suggested that TT subjects need at least 10% more dietary folate than CT and CC subjects to achieve a comparable high plasma folate level and a comparable low homocysteine level.

In **CHAPTER 6** we described the relation between coffee, tea and alcohol consumption, physical activity and smoking and the homocysteine concentration. Cross-sectional information provided by the random sample (n=2601) was used for this goal. The lifestyle factors most

strongly associated with the homocysteine concentration were coffee consumption (positive association), smoking (positive association), and alcohol consumption (inverse association). We did not observe a statistically significant association between tea drinking and homocysteine. Furthermore, physical activity was weakly positively associated with homocysteine in women only. Independently of other lifestyle factors, age, folate and B-vitamin supplement intake drinking of more than 6 cups of coffee per day was associated with a 1.4 µmol/L higher homocysteine concentration in men and a 1.1 µmol/L higher homocysteine concentration in women, compared with non-drinkers. The smoking effect was most prominent in women; in non-smokers the homocysteine concentration was 0.8 µmol/L lower than in smokers of more than 20 cigarettes per day. The alcohol effect was most pronounced in men. In men with a folate intake below the median (204 µg per day), the homocysteine concentration in non-drinkers was 1.7 µmol/L higher compared with drinkers of more than 2 glasses of alcohol per day. In men with a folate intake above the median, this difference in homocysteine was 0.8 µmol/L. From these results we concluded that changes in lifestyle factors may influence the homocysteine concentration.

The inverse relation between alcohol consumption and homocysteine in men was further investigated in **CHAPTER 7**. We distinguished between three types of alcoholic beverages: beer, wine and spirits. Independently of other lifestyle factors and B-vitamin intake, beer drinking was associated with a lower homocysteine concentration. Men drinking no beer had a homocysteine concentration of 14.3 µmol/L, whereas men drinking on average 3.7 glasses of beer per day, had a homocysteine concentration of 13.0 µmol/L. Drinking wine or spirits did not have an effect on the homocysteine concentration, possibly due to the small range of intake relative to the range of beer consumption. The observed association between beer and homocysteine can probably not be ascribed to folate, and other B-vitamins present in beer, as we adjusted for these effects. This might indicate that ethanol intake is inversely associated with the homocysteine concentration. We could not properly investigate this, as beer was the predominant drink (i.e. ethanol provider) in this population.

In **CHAPTER 8** we evaluated to what extent biological coronary heart disease risk factors are associated with the homocysteine concentration. In this study we used data of the random sample on total and HDL cholesterol levels, waist circumference and diastolic and systolic blood pressure. Total and the HDL cholesterol levels were associated with the homocysteine concentration in women only, independently of lifestyle factors, other biological coronary heart disease risk factors and folate intake. The other biological coronary heart disease risk factors were not associated with the homocysteine concentration. Women with a total cholesterol level in the lowest quartile had a homocysteine concentration 0.6 µmol/L lower than that of women

with a total cholesterol level in the highest quartile. The association between HDL cholesterol and homocysteine was inverse: women with a HDL cholesterol level in the lowest quartile had a homocysteine concentration 1 µmol/L higher than that of women in the highest HDL quartile. Although not all biological coronary heart disease risk factors were statistically significantly associated with the homocysteine concentration, it remains necessary to evaluate their confounding effect when the relation between homocysteine and coronary heart disease is investigated; because the relation between biological coronary heart disease risk factors and homocysteine is in the direction of an increased risk of coronary heart disease.

Results of prospective studies that consider the relation between the homocysteine concentration and the risk of coronary heart disease have been inconsistent. The same applies to prospective studies that investigated the relation between plasma B-vitamins (inversely associated with the homocysteine concentration) and coronary heart disease. To provide more insight into this topic we determined the relation between plasma homocysteine, folate, vitamin B6 (PLP) and B12 and coronary heart disease mortality in **CHAPTER 9**. This study was done within a population-based cohort of ~36,000 Dutch adults aged 20-59 years at baseline, who were followed for an average period of 10.3 years. The design was a case-cohort study, using a randomly drawn reference cohort of 630 subjects to which all coronary heart disease (n=102) cases, who were not part of the reference cohort, were added. All these subjects were free of cardiovascular diseases at baseline.

After correction for age, sex, study center, hypertension, HDL and total cholesterol, smoking and creatinine level we found that each 5 µmol/L increase in homocysteine was associated with a statistically non-significant increased risk of 3% for coronary heart disease mortality (relative risk (RR)=1.03, 95% confidence interval (CI)=0.83-1.29). In women only, high folate levels were associated with a statistically significant protection of coronary heart disease mortality; each 5 nmol/L increase in plasma folate concentration was associated with a RR of 0.16 (95% CI=0.03-0.78). Plasma PLP and vitamin B12 concentrations were not associated with coronary heart disease mortality. From these results we concluded that elevated homocysteine levels are not a major risk factor for coronary heart disease in this population sample of relatively young healthy Dutch subjects. In women, a higher plasma folate concentration might reduce the risk of coronary heart disease.

In the last chapter of this thesis (**CHAPTER 10**) we evaluated the results described in this thesis in light of other epidemiological studies. We focussed on methodological issues and we evaluated to what extend there is proof that elevated tHcy concentrations are a causal risk factor for coronary heart disease.

For the most important homocysteine determinants, i.e., folate intake, coffee consumption, and the 677 C>T polymorphism of the MTHFR gene, our results agree with those of other epidemiological studies. Our results suggest an inverse association between alcohol consumption and homocysteine; this needs further study as in other epidemiological studies alcohol consumption was associated with higher homocysteine concentrations. The relation between smoking and homocysteine needs to be studied in depth because other lifestyle factors that are difficult to take into account may be responsible for the positive association between smoking and a high homocysteine concentration.

Our result that an increased tHcy concentration is not an important risk factor for coronary heart disease agrees with 8 of the 17 published prospective studies on this topic. The reason for our lack of a statistically significant association could be due to methodological aspects: e.g. the blood handling and storage conditions of this study were not optimal for the homocysteine measurement and this might have diluted the association between homocysteine and coronary heart disease mortality. However, a more likely explanation is provided by the mechanism by which homocysteine might increase the risk of coronary heart disease. Some researchers have hypothesized that homocysteine is a short-term risk marker that exacerbates the effects of other coronary heart disease risk factors (like hypertension and hypercholesterolemia), and thereby promotes an acute coronary heart disease event. If this is true, homocysteine is probably not a major risk factor in a study with relatively young subjects free of baseline cardiovascular diseases studied after a long follow-up. This hypothesis might also explain some of the inconsistencies in the results of published prospective studies. Yet, the hypothesis is mainly based on prospective studies with high-risk subjects (e.g. subjects with coronary heart disease). This makes it difficult to exclude the possibility that the increased homocysteine levels are the consequence of vascular damage present in these subjects.

The only studies that can provide definitive answers for the causality question, are intervention studies. There are several ongoing secondary intervention trials, in which high-risk subjects are supplemented with folic acid, vitamin B6 and B12. If these trials show that homocysteine-lowering through vitamin supplementation, prevents coronary heart disease in the treatment groups, this makes a strong case for the hypothesis that homocysteine is the causative agent that leads to coronary heart disease in these high-risk subject. Note, however, that if the incidence of coronary heart disease decreases in these trials, this could also be the consequence of a higher intake of B-vitamins. Other intervention studies with different homocysteine-lowering compounds (like betaine) may provide insight into whether a lower homocysteine or a higher B-vitamin intake is responsible for the protection against coronary heart disease. Primary intervention trials with apparently healthy subjects (e.g. no cardiovascular diseases, no diabetes) with elevated homocysteine levels, are the only studies

that can elucidate whether homocysteine is a causal risk factor for coronary heart disease in healthy subjects.

In conclusion, elevated homocysteine levels are probably not a major risk factor for coronary heart disease in healthy subjects. Yet, increased homocysteine concentrations are consistently associated with an increased risk of coronary heart disease in high-risk subjects. Thus, with regard to implications for prevention and treatment, we agree with the recent guidelines of the Netherlands Heart Foundation that subjects at high risk of cardiovascular diseases may be screened for elevated homocysteine levels. If elevations are observed, treatment with 500 µg of folic acid per day can be applied under strict conditions. This will lower their homocysteine concentration, and may have a beneficial effect on their risk of coronary heart disease. For healthy subjects it remains important to follow the guidelines of health authorities to increase the consumption of foods of plant origin. This will increase the intake of folate and simultaneously the intake of other compounds which have a beneficial effect on health in general.

SAMENVATTING

Hart- en vaatziekten, en in het bijzonder coronaire hartziekten, vormen de belangrijkste doodsoorzaak in geïndustrialiseerde landen. De belangrijkste risicofactoren voor het ontstaan van coronaire hartziekten zijn: een hoog totaal cholesterolgehalte en/of een laag HDL-cholesterolgehalte in het bloed, roken en een hoge bloeddruk. Verhoogde bloed homocysteïne concentraties zijn recent ook in verband gebracht met een verhoogd risico op coronaire hartziekten.

Homocysteïne is een aminozuur dat niet gebruikt wordt voor de opbouw van eiwitten en nauwelijks voorkomt in de voeding. Het ontstaat intracellulair wanneer het aminozuur methionine wordt gemetaboliseerd. De intracellulaire concentratie van homocysteïne wordt exact gereguleerd. Een teveel aan homocysteïne in de cel wordt naar het bloedplasma getransporteerd. Aldaar zouden verhoogde concentraties mogelijk atherosclerose en/of trombose kunnen stimuleren.

Er is nog geen overtuigend bewijs dat het verband tussen een verhoging van het homocysteïnegehalte en coronaire hartziekten causaal is. Indien deze relatie causaal is, dan zou een verlaging van de homocysteïneconcentratie een daling van het aantal gevallen van coronaire hartziekten tot gevolg hebben. Het is dan ook belangrijk om te weten welke factoren het homocysteïnegehalte bepalen.

In dit proefschrift zijn onderzoeken beschreven die voedingsgewoonten, leefstijl, genetische factoren en biologische risicofactoren voor coronaire hartziekten onderzocht hebben als mogelijke determinanten van het homocysteïnegehalte. Deze studies werden gedaan in een grootschalig dwarsdoorsnede-onderzoek. Daarnaast is een prospectief-onderzoek beschreven waarin het risico gekwantificeerd is op het sterven aan coronaire hartziekten ten gevolge van een verhoogde homocysteïne concentratie. De onderzoekspopulaties van het dwarsdoorsnede- en het prospectief-onderzoek waren representatief voor de Nederlandse bevolking (20-65 jaar oud).

Het is al enige decennia bekend dat bij patiënten met erfelijke afwijkingen in het homocysteïnemetabolisme de extreem hoge homocysteïneconcentratie verlaagd kan worden met foliumzuur. Foliumzuur is de synthetische vorm van folaat die in vitaminepreparaten aanwezig is. Daarentegen was het de vraag of een hogere folaatinname (uit de voeding en/of supplementen) de homocysteïneconcentratie in de algemene bevolking zou kunnen beïnvloeden. De stand van zaken omtrent dit vraagstuk ten tijde van 1997 is samengevat in **HOOFDSTUK 2**. In dit literatuuroverzicht beschreven we de daadwerkelijke, de aanbevolen en de

wenselijke inname van folaat. Bij het zoeken naar literatuur beperkten wij ons vooral tot studies die informatie gaven over Europese volwassenen. Aangezien niet bekend was vanaf welke homocysteïneconcentratie een risico ontstaat op coronaire hartziekten en aangezien aangenomen werd dat het risico continue daalt met het afnemen van het homocysteïne gehalte, werd de wenselijke folaatinname geschat met het oog op een zo laag mogelijk homocysteïnegehalte.

De belangrijkste conclusies van dit literatuuronderzoek waren dat de daadwerkelijke folaatinname in Europa (mannen: 291, vrouwen: 247 µg per dag) overeenkwam met de aanbevelingen (170-300 µg per dag). Echter de geconsumeerde en aanbevolen hoeveelheid folaat was lager dan het wenselijke niveau van meer dan 350 µg per dag, dat nodig zou zijn om een zo laag mogelijke homocysteïneconcentratie te bereiken.

Indien een verhoogd homocysteïnegehalte een causale risicofactor is voor het ontwikkelen van coronaire hartziekten, dan is het voor instanties die zich richten op de volksgezondheid van groot belang te beschikken over een goede schatting van het aantal mensen dat een verhoogd homocysteïnegehalte heeft. Uit een homocysteïneverdelingscurve kan worden afgelezen hoeveel procent van de populatie een homocysteïneconcentratie heeft boven een bepaalde grenswaarde. De keuze van deze grenswaarde is relatief arbitrair waneer wordt aangenomen dat het verband tussen het homocysteïnegehalte en coronaire hartziekten continue is. Echter vanuit praktisch oogpunt is het nodig een grenswaarde te hanteren. Een homocysteïneverdelingscurve voor Nederlandse volwassenen werd weergegeven in **HOOFDSTUK 3**. Hiervoor bepaalden we het niet-nuchtere homocysteïnegehalte in een aselecte steekproef van 3025 mannen en vrouwen van 20-65 jaar. De gemiddelde homocysteïneconcentratie was 14,6 (minimum 5,9 en maximum 94,6) µmol/L voor mannen en 13,1 (minimum 6,1 en maximum 72,2) µmol/L voor vrouwen.

Een vooraanstaand laboratorium in Bergen, Noorwegen hanteert 15 µmol/L als grenswaarde voor een verhoogde homocysteïneconcentratie. Deze waarde konden wij niet eenvoudigweg overnemen omdat er tussen laboratoria grote verschillen zijn in de gemeten homocysteïneconcentratie van één plasmamonster. Om een vergelijking mogelijk te maken zijn de homocysteïnewaarden van 301 plasmamonsters in ons laboratorium in Nijmegen en in Bergen gemeten. Het resultaat gaf aan dat de waarden in Nijmegen systematisch 2,4 µmol/L hoger lagen. Rekening houdend met dit verschil was het percentage met een verhoogde homocysteïneconcentratie (>17,4 µmol/L) in Nederlandse mannen 14,4% en in vrouwen 8,9%. In vergelijking met de gegevens van andere Europese landen waarvoor het homocysteïnegehalte in Bergen werd gemeten, waren de Nederlandse waarden na correctie voor het systematische verschil over het algemeen hoger.

Zoals eerder vermeld, waren er weinig gegevens over de relatie tussen folaat uit de voeding en het homocysteïnegehalte. Bovendien was weinig bekend over de relatie tussen het homocysteïnegehalte en de inname van andere B-vitamines die essentieel zijn voor het metabolisme van homocysteïne. Om die reden onderzochten wij (**HOOFDSTUK 4**) de relatie tussen de inname van folaat, riboflavine (vitamine B2), vitamine B6 and B12 en de homocysteïneconcentratie. Voor dit onderzoek maakten we gebruik van een subgroep (n=2435) van de hierboven beschreven aselecte steekproef. Een hogere inname van B-vitamines ging samen met een lagere homocysteïneconcentratie. Wanneer echter gecorrigeerd werd voor mogelijk verstorende variabelen (inname van andere B-vitamines, inname van methionine, leeftijd, roken en het drinken van alcohol) bleek dat alleen de folaatinname invers geassocieerd was met de homocysteïneconcentratie. Een gemiddeld lage folaatinname (mannen: 161, vrouwen 160 µg per dag) ging samen met een hoge homocysteïneconcentratie: bij mannen gemiddeld 15,4 µmol/L en bij vrouwen gemiddeld 13,7 µmol/L. Een gemiddeld hoge folaatinname (mannen: 254, vrouwen 262 µg per dag) ging samen met een lage homocysteïnewaarde: bij mannen gemiddeld 13,2 µmol/L en bij vrouwen gemiddeld 12,4 µmol/L. Bij mannen was het verschil in homocysteïnegehalte tussen een lage en hoge folaatinname groter bij rokers dan bij niet-rokers (2,8 vs. 1,6 µmol/L). Dit was ook het geval bij mannen die geen alcohol dronken vergeleken met mannen die meer dan 2 alcoholische dranken per dag gebruikten (3,5 vs. 1,4 µmol/L). Wij concludeerden dat in de Nederlandse bevolking folaat als enige B-vitamine invers geassocieerd was met de homocysteïneconcentratie. Een hogere folaatinname via de voeding zou dus een verlagend effect kunnen hebben op de homocysteïneconcentratie.

Naast B-vitamines is ook de activiteit van enzymen die een rol spelen in het homocysteïnemetabolisme van belang voor het homocysteïnegehalte. Het enzym 5,10-methyleen-tetrahydrofolaatreductase (MTHFR) produceert de belangrijkste circulerende vorm van folaat die nodig is voor de omzetting van homocysteïne naar methionine. Een mutatie in het gen dat codeert voor MTHFR, het 677 C>T polymorfisme, vermindert de enzymactiviteit. De enzymactiviteit is normaal bij personen met het CC-genotype, enigszins verminderd bij personen met het CT-genotype en duidelijk verlaagd bij personen met het TT-genotype. In **HOOFDSTUK 5** beschreven we de consequenties van dit genotype voor de folaatinname, het plasmafolaatgehalte en het homocysteïnegehalte. Opnieuw voerden we een dwarsdoorsnede-onderzoek uit bij een subgroep (n=2051) van de aselecte steekproef. Het verband tussen folaatinname en de plasmafolaatconcentratie was positief voor alle genotypen. De sterkste positieve relatie was te zien bij personen met het CT-genotype: bij een lage folaatinname (gemiddeld 155 µg per dag) was de plasmafolaatconcentratie van personen met het CT-genotype vergelijkbaar met die van personen met het TT-genotype (CT: 5,8; TT: 5,7 nmol/L),

echter bij een hogere folaatinname (gemiddeld 267 µg per dag) was de plasmafolaat concentratie gelijk aan die van personen met het CC-genotype (CT: 8,4; CC: 8,7 nmol/L). Bij personen met het TT-genotype bleek de plasmafolaatconcentratie altijd lager te zijn dan die van personen met het CT- en CC-genotype ondanks gelijke folaatinname. De relatie tussen het plasmafolaatgehalte en het homocysteïnegehalte was invers voor alle genotypen en het sterkst bij personen met het TT-genotype: bij een lage plasmafolaatconcentratie (gemiddeld 4,0 nmol/L) was de homocysteïneconcentratie veel hoger dan voor de andere genotypen (TT: 20,9, CT: 15,4; CC: 14,3 µmol/L), terwijl bij een hoge folaatconcentratie (13,7 nmol/L) het verschil in homocysteïneconcentratie tussen de genotypen verwaarloosbaar was. De relatie tussen folaatinname en het homocysteïnegehalte was eveneens invers en het duidelijkst bij personen met het TT-genotype. Onze resultaten wezen erop dat personen met het TT-genotype op zijn minst 10% meer folaat uit de voeding nodig hebben dan personen met het CT- en CC-genotype om een vergelijkbaar hoog plasmafolaatniveau en een vergelijkbaar laag homocysteïneniveau te verkrijgen.

In **HOOFDSTUK 6** beschreven we de relatie tussen de leefstijlfactoren koffie-, thee- en alcoholconsumptie, lichamelijke activiteit en roken en de homocysteïneconcentratie. We gebruikten de cross-sectioneel verkregen informatie van 2601 personen uit de aselecte steekproef. De leefstijlfactoren die het sterkst gerelateerd waren aan de homocysteïneconcentratie waren koffieconsumptie (positief verband), roken (positief verband) en alcoholconsumptie (invers verband). Er was geen statistisch significant verband tussen het drinken van thee en de homocysteïneconcentratie. Het verband tussen lichamelijke activiteit en het homocysteïnegehalte was alleen bij vrouwen zwak positief. Onafhankelijk van andere leefstijlfactoren, leeftijd, folaatinname, en inname van B-vitamine supplementen was het drinken van meer dan 6 kopjes koffie per dag geassocieerd met een 1,4 µmol/L hogere homocysteïneconcentratie bij mannen en een 1,1 µmol/L hogere homocysteïneconcentratie bij vrouwen, in vergelijking tot personen die geen koffie drinken. Het effect van roken was het sterkst bij vrouwen. Bij de niet-rokende vrouwen was de homocysteïneconcentratie 0,8 µmol/L lager dan bij vrouwen die meer dan 20 sigaretten per dag rookten. Het effect van alcoholconsumptie was het duidelijkst bij mannen. Bij mannen met een folaatinname lager dan de mediaan (204 µg per dag), was de homocysteïneconcentratie bij niet-drinkers 1,7 µmol/L hoger dan bij mannen die meer dan 2 glazen alcohol per dag dronken. Bij mannen met een folaatinname boven de mediaan was dit verschil in homocysteïneconcentratie 0,8 µmol/L. Op basis van deze resultaten concludeerden wij dat een verandering in leefstijl het homocysteïnegehalte kan beïnvloeden.

Het inverse verband tussen alcoholconsumptie en het homocysteïnegehalte bij mannen werd nader bestudeerd in **HOOFDSTUK 7**. Hier maakten we onderscheid tussen drie soorten alcoholische dranken: bier, wijn en sterke drank. Onafhankelijk van andere leefstijlfactoren en de inname van B-vitamines, was het drinken van bier geassocieerd met een lagere homocysteïneconcentratie. Mannen die geen bier dronken hadden een homocysteïneconcentratie van 14,3 µmol/L, terwijl mannen die gemiddeld 3,7 glazen bier per dag dronken een homocysteïneconcentratie van 13,0 µmol/L hadden. Het drinken van wijn of sterke drank had geen effect op de homocysteïneconcentratie. Dit komt mogelijk door de beperkte consumptie van dit soort dranken in vergelijking met die van bier. Het feit dat bier folaat en andere B-vitamines bevat, kan de gevonden relatie waarschijnlijk niet verklaren omdat we voor deze effecten gecorrigeerd hadden. Dit zou er op kunnen wijzen dat de inname van ethanol verantwoordelijk is voor het inverse verband met de homocysteïneconcentratie. Dit konden wij niet nader onderzoeken, aangezien bier in onze onderzoekspopulatie de belangrijkste bron van ethanol was.

In **HOOFDSTUK 8** zijn we nagegaan in hoeverre biologische risicofactoren voor coronaire hartziekten geassocieerd zijn met de homocysteïneconcentratie. Voor dit onderzoek maakten we gebruik van de gegevens over het totaal- en HDL-cholesterolgehalte, de systolische en diastolische bloeddruk en de buikomtrek van de aselecte steekproef. Alleen bij vrouwen bleek het totaal en het HDL-cholesterolgehalte geassocieerd met de homocysteïneconcentratie, ook ná correctie voor leefstijlfactoren, andere biologische risicofactoren voor coronaire hartziekten en folaatinname. De andere biologische risicofactoren waren niet geassocieerd met de homocysteïneconcentratie. Vrouwen met een totaal cholesterolgehalte in het laagste kwartiel hadden een homocysteïneconcentratie die 0,6 µmol/L lager was dan die van vrouwen met een totaal cholesterolgehalte in het hoogste kwartiel. De associatie tussen het HDL-cholesterolgehalte en het homocysteïnegehalte was invers: vrouwen met een HDL-cholesterolgehalte in het laagste kwartiel hadden een homocysteïneconcentratie die 1 µmol/L hoger was dan bij vrouwen in het hoogste HDL-kwartiel. Ofschoon niet alle biologische risicofactoren statistisch significant geassocieerd waren met de homocysteïneconcentratie, blijft het van belang om het effect van deze factoren te evalueren. Immers, de resultaten wijzen op een verband tussen biologische risicofactoren en het homocysteïnegehalte in de richting van een verhoogd risico op coronaire hartziekten.

De resultaten van prospectieve studies omtrent de relatie tussen het homocysteïnegehalte en de kans op coronaire hartziekten zijn niet consistent. Dit is ook het geval voor prospectieve studies die gekeken hebben naar de associatie tussen de plasma concentraties van B-vitamines (invers geassocieerd met de homocysteïneconcentratie) en de kans op coronaire hartziekten.

Om meer inzicht in deze relaties te krijgen onderzochten wij het verband tussen enerzijds de plasmaconcentraties van homocysteïne, folaat, vitamine B6 (PLP) en vitamine B12 en anderzijds de sterfte aan coronaire hartziekten in **HOOFDSTUK 9**. Dit onderzoek werd uitgevoerd met gegevens van een cohort van ruim 36.000 mannen en vrouwen (20-59 jaar) wat gevolgd werd gedurende een periode van 10,3 jaar gemiddeld. Dit onderzoek had een zogenaamd "case-cohort" opzet. Dit wil zeggen dat we van het totale cohort alleen gebruik maakten van de gegevens van een aselecte steekproef van 630 deelnemers en van alle 102 sterfgevallen aan coronaire hartziekten. Alle personen hadden bij aanvang van het onderzoek geen hart- en vaatziekten.

Na correctie voor leeftijd, geslacht, onderzoekscentrum, hypertensie, HDL- en totaal cholesterol, roken en de kreatinineconcentratie constateerden we dat elke 5 µmol/L stijging van het homocysteïnegehalte geassocieerd was met een statistisch niet-significante stijging van 3% in de kans op sterfte aan coronaire hartziekten (Relatief Risico (RR): 1,03; 95% betrouwbaarheidsinterval (BI)=0,83-1,29). Verder vonden we dat uitsluitend voor vrouwen gold dat een hogere plasmafolaatconcentratie geassocieerd was met een statistisch significante bescherming tegen coronaire hartziekten: elke 5 nmol/L stijging van de plasmafolaatconcentratie was geassocieerd met een RR van 0,16 (95% BI=0,03-0,78). De plasmaconcentraties van PLP en vitamine B12 waren niet geassocieerd met het risico van sterfte aan coronaire hartziekten. We concludeerden dat een verhoogde homocysteïneconcentratie geen belangrijke risicofactor is voor sterfte aan coronaire hartziekten in deze populatie van relatief jonge Nederlandse personen. Bij vrouwen zou een hogere plasmafolaatconcentratie mogelijk het risico van coronaire hartziekten kunnen verlagen.

In **HOOFDSTUK 10** zijn de resultaten van dit proefschrift besproken in het licht van andere epidemiologische bevindingen. Hierbij kregen methodologische aspecten aandacht en verder werd geëvalueerd in hoeverre er bewijs bestaat dat een verhoging van het homocysteïnegehalte een oorzakelijke rol speelt in het ontstaan van coronaire hartziekten.

Van de onderzochte factoren waren de folaatinname, koffieconsumptie en het 677 C>T polymorfisme in het MTHFR-gen de belangrijkste determinanten van het homocysteïnegehalte. Dit komt overeen met de resultaten van andere epidemiologische onderzoeken. Meer onderzoek is nodig naar de mogelijk inverse relatie tussen alcoholconsumptie en de homocysteïneconcentratie aangezien er ook studies zijn die een positief verband rapporteren. Ook de relatie tussen roken en de homocysteïneconcentratie zou nader bestudeerd moeten worden, omdat andere leefstijlfactoren, waarvoor moeilijk te corrigeren is, mogelijk verantwoordelijk zijn voor het gevonden positieve verband tussen roken en een hoge homocysteïneconcentratie.

Onze bevinding dat verhogingen in het homocysteïnegehalte geen belangrijke risicofactor zijn voor het sterven aan coronaire hartziekten komt overeen met 8 van de 17 gepubliceerde prospectieve studies over hetzelfde onderwerp. De reden dat wij geen statistisch significant verband hebben gevonden kan liggen aan methodologische aspecten van ons onderzoek; de condities van bloedafname en opslag waren niet optimaal voor de homocysteïnebepaling. Dit kan hebben geleid tot een afzwakking van het verband tussen de homocysteïneconcentratie en sterfte aan coronaire hartziekten. Het lijkt echter waarschijnlijker dat de verklaring ligt in het mechanisme waarmee verhogingen in de homocysteïneconcentratie de kans op coronaire hartziekten verhogen. Een aantal onderzoekers heeft namelijk geopperd dat een verhoogde homocysteïneconcentratie een korte-termijn risicofactor is, die het effect van andere risicofactoren voor coronaire hartziekten (zoals hypertensie en hypercholesterolemie) verergert, en op deze manier een acuut coronair incident induceert. Wanneer deze hypothese klopt dan is het logisch dat een verhoging van het homocysteïnegehalte geen belangrijke risicofactor is in een langlopend prospectief onderzoek met relatief jonge personen zonder hart- en vaatziekten. Het voorgestelde werkingsmechanisme lijkt ook een deel van de verschillen tussen uitkomsten van alreeds gepubliceerde prospectieve studies te verklaren. De hypothese dat homocysteïne een korte-termijn risicofactor is, is echter voornamelijk gebaseerd op prospectieve onderzoeken met personen die geselecteerd waren op hun hoge risico voor het krijgen van hart- en vaatziekten. Deze laten consistent een sterk verband zien tussen het homocysteïnegehalte en de kans op coronaire hartziekten. De moeilijkheid bij dit soort onderzoeken is dat niet is uit te sluiten dat het verhoogde homocysteïnegehalte een gevolg is van reeds aanwezige vaatschade in deze personen.

De enige onderzoeken die een antwoord op het causaliteitsvraagstuk kunnen geven zijn interventiestudies. Momenteel worden verschillende secundaire interventiestudies uitgevoerd waarin personen met een hoog risico op hart- en vaatziekten supplementen met foliumzuur, vitamine B6 and B12 toegediend krijgen. Indien deze interventiestudies aantonen dat een homocysteïneverlaging door deze supplementen gepaard gaat met minder gevallen van coronaire hartziekten, dan vormt dit een sterk bewijs voor de hypothese dat het homocysteïnegehalte een factor is die coronaire hartziekten veroorzaakt bij hoog-risico personen. Het is echter wel zo dat een daling in het aantal gevallen van coronaire hartziekten ook (mede) het gevolg kan zijn van een hogere inname van B-vitamines. Interventiestudies met andere homocysteïneverlagende stoffen (zoals betaïne) zouden de vraag kunnen beantwoorden of bescherming tegen coronaire hartziekten een gevolg is van de verlaging van het homocysteïnegehalte, of van de verhoging van de inname van B-vitamines. Interventiestudies bij ogenschijnlijk gezonde personen (dat wil zeggen zonder hart- en vaatziekten, diabetes, hypertensie, etc.) met een verhoogd homocysteïnegehalte, zijn de enige studies die kunnen

aantonen of er een causale relatie bestaat tussen het homocysteïnegehalte en coronaire hartziekten bij gezonde personen.

Concluderend kan gesteld worden dat een verhoogde homocysteïneconcentratie geen belangrijke risicofactor zijn voor het krijgen van coronaire hartziekten bij gezonde personen. Echter, bij hoog-risico personen zijn verhogingen in het homocysteïnegehalte wel consistent geassocieerd met een verhoogd risico op coronaire hartziekten. Daarom sluiten we ons aan bij de recente richtlijnen van de Nederlandse Hartstichting die adviseert om bij personen met een hoog risico op hart- en vaatziekten het homocysteïnegehalte te bepalen. Als het homocysteïnegehalte verhoogd is, kan onder strikte voorwaarden behandeling met 500 µg foliumzuur per dag worden toegepast. Extra foliumzuur zal tot een daling van het homocysteïnegehalte leiden en mogelijk tot een daling van de kans op het krijgen van coronaire hartziekten. Voor gezonde personen blijft het belangrijk om tenminste de aanbevolen hoeveelheid van twee ons groenten en twee stuks fruit per dag, op te volgen. Het gebruik van meer plantaardige producten gaat gepaard met een hogere folaatinname en tegelijkertijd ook met een hogere inname van andere plantaardige stoffen die een positief effect op de gezondheid hebben.

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ABOUT THE AUTHOR

Angelika de Bree was born on May 23, 1972 in Eemnes, the Netherlands. After completing secondary school in 1990 (VWO at the Gooisch Lyceum in Bussum) she studied Human Nutrition at the Wageningen University. As a part of this study she conducted epidemiological research projects at the department of Internal Medicine at the University Medical Center St Radboud in Nijmegen, at the former department of Epidemiology and Public Health of the Wageningen University and at the unit of Nutrition and Cancer of the International Agency for Research on Cancer, in Lyon, France. In July 1996 she received her MSc degree.

In July 1996 she started a research project on folate intake and homocysteine and their relation with neural tube defects, cardiovascular diseases and cancer for Unilever Research Laboratory Vlaardingen in collaboration with the Department of Human Nutrition of the Wageningen University. In April 1997 she started the PhD-project described in this thesis. This project was a collaboration between the Department of Chronic Diseases Epidemiology of the National Institute of Public Health in Bilthoven, and the laboratory of Pediatrics and Neurology of the University Medical Center St Radboud. During this project she participated in numerous courses among which the genetic epidemiological courses organized by the Erasmus Summer Programme at the Erasmus University Medical School, Rotterdam, and epidemiological courses organized by New England Epidemiology Institute at Tufts University, Boston, USA. She worked in the laboratories of the department of Pharmacology of the University of Bergen in Norway (Prof. P.M. Ueland, Prof. H. Refsum) and of the USDA Human Nutrition Center on Aging at Tufts University in Boston, USA (Prof. J. Selhub) for the determination of vitamin B12 and folate concentrations. She participated in the European Nutrition Leadership Programme 2001 in Luxembourg. During her PhD-project she performed a project of three months on homocysteine for Unilever Research Laboratory Vlaardingen.

In February 2002 she will start working as a Post-doc at INSERM unit 557, INRA unit 1125, Scientific and Technical Institute of Nutrition and Food (ISTNA) of Prof. S. Hercberg in Paris, France, where she will work on the relation between homocysteine metabolism and inflammation and hemostasis.